Characterisation of the Prokaryotic community of Lake Suigetsu, Japan: towards a novel palaeoenvironment research biomarker

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Abstract

Sediment cores from Lake Suigetsu, Japan are recognised as a key record of past climate reconstruction because of the finely laminated sediments that provide precise event stratigraphy. Here, we study the relationship between the microbial communities in the lake sediments of Lake Suigetsu during validated episodes of environmental change.

We use fossil DNA from the lake sediment and utilising the PCR-DGGE technique, we detected the presence of several taxa. Among the investigated sediment cores, the *Acidobacteria* community was found to be the most abundant while the *Actinobacteria* community was the least. The results showed that the overall bacterial community structure and their diversity were significantly affected by sediment depths, rather than the availability of nutrient (i.e. TOC and TN).

The first event was the introduction of saline water in Lake Suigetsu. Historical records have described this event occurring during 1664 AD, which equates to the sediment depth of approximately 81.64 cm. A metagenomics study based on selected sediment depths has exhibited a shift in the bacterial taxa, consistent with the transition of lake salinity from freshwater to brackish. *Bacillaceae* and *Clostridiaceae* were found to be more predominant in the brackish sediments relative to the freshwater sediments. Evidence of the seawater incursion was found in the sediment depths between 82.16 and 83.16 cm.

The second event was a climate event dated back to the Late Quaternary period. The results presented here show that bacterial diversity and species richness become increased when climate changed from a cold to warmer conditions. The metagenomics analysis on the sediment deposits has demonstrated distinctive differentiations in bacterial taxa during the climate transition from the colder to warmer episodes. This observation could be related to the rapid adaptation/tolerance of bacteria to environmental changes, or simply the effect of depth. Although the temperature-dependent $\delta^{15}N$ isotope can be strongly correlated to the bacterial communities, the weak selectivity of the $\delta^{15}N$ isotope could result in false correlation between the $\delta^{15}N$ isotope and the diversity of the bacterial communities.

The application of molecular and culture-dependent techniques was used to characterise bacterial diversity in the sedimentary records of Lake Suigetsu. The culture-based techniques showed a better representation of high GC *Actinobacteria* while molecular techniques revealed a better profile of Gram negative bacteria. Furthermore, based on a polyphasic approach, several putatively new species have been identified, notably *Actinobacteria* strains that belong to the genera *Dermacoccus*, *Dietzia*, *Leifsonia* and *Rhodococcus.* Among the tested strains, a novel *Rhodococcus* isolate that was recovered from the freshwater sediment, merits recognition of new species status and the name *Rhodococcus meromictica* sp. nov is proposed.

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Declaration

I declare that the work contained in this thesis is all my own work and it has never been submitted or approved for any other award by this or any other university.

Name:

Signature:

Date:

Abbreviations

1D/2D-TLC	One/Two dimensional thin layer chromatography
AD	Anno Domini
AMS	Accelerator mass spectrometry
APS	Ammonium persulphate
ATP	Adenosine triphosphate
a.p.s.l.	Above present sea level
$BioSiO_2$	Biogenic silica
BP	Before present
bp	Base pair
Bølling	Bølling-Allerød
BSA	Bovine serum albumin
BLAST	Blast local alignment search tool
ca.	Circa. (about/approximately)
cal	Calibrated years/Calendar years before 1950
Ca^{2+}	Calcium ions
$CaCO_3$	Calcium carbonate
Cl^-	Chlorine ions
CO_2	Carbon dioxide
CO_3^-	Carbon trioxide ions
CO_3^2	Carbonate
^{12}C	Carbon-12
$^{12}CO_2$	Carbon dioxide-12
^{14}C	Carbon-14
CCA	Canonical correspondence analysis
COMX	Cool mixed forest

$\delta^{15}N$	delta-N-15
DCA	Detrended correspondence analysis
DDC	Dewey decimal classifcation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FAMES	Fatty acids methyl esters
F’	Forward
Fe^{2+}	Iron (II)
Fe_2O_3	Iron (III) oxide
g	Gram(s)
× g	Gravity
GC	Guanine-Cytosine
GC-MS	Gas chromatography-mass spectrometry
GI	Greenland interstadial
H’	Shannon-Wiener Index
H_2S	Hydrogen sulphide
HCO_3^-	Bicarbonate ions
INQUA	International Union for Quaternary Research
INTIMATE	Integration of Ice-core, Marine and Terrestrial records
K^+	Potassium ions
ka	kiloannum, a unit of time equal to one thousand (10^3) years
Kb	Kilobase
km	kilometre
km^2	kilometre square
Mg^{2+}	Magnesium ions
mm	millimetre

Na^+	Sodium ions
NERC	Natural Environment Research Council
NO_3^-	Nitrate
NH_4^+	Ammonia
N_2	Nitrogen
m	Metre (s)
M	Molar
MA	Marine agar
mA	Milliamps
$MgSO_4$	Magnesium sulphate
OTU	Operational taxonomic unit
^{206}Pb	Lead 206
^{210}Pb	Lead 210
PO_4^{3-}	Phosphate
PC1	Principal Component 1
PC2	Principal Component 2
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
PLS-DA	Principle Least Square Discriminant Analysis
Q-PCR	Quantitative PCR
^{222}Rn	Radon 222 isotope
R’	Reverse
RDA	Redundancy analysis
Rr	Species richness
rDNA	ribosomal DNA
RNA	Ribonucleic acid
rRNA	ribosomal RNA

SO_4^{2-}	Sulphate ions
SG	Suigetsu
SRB	Sulphate-reducing bacteria
TAE	Tris-acetate-EDTA
TEDE	Temperate deciduous forest
TEMED	N,N,N',N'-Tetramethylethylenediamine
TN	Total nitrogen
TOC	Total organic carbon
TPA	Trypticase peptone yeast agar
T-RFLP	Terminal Restriction Fragment Length Polymorphism
U	Units
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WAMX	Warm mixed forest
yr	Year

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Chapter 1 Introduction

1.1. Background

Large lakes are globally distributed providing network of sites that can be used to assess regional variations in climate change across continents. They are 'sentinel systems', for environmental and climate change, recording signals from both regional and global climate forcing (Battarbee, 2000; Lake *et al*., 2000; Woodward *et al*., 2010). While, lakes sediments are suitable for climate change reconstruction the lakes themselves vary in origin, geographical distribution, age, and chemistry (Battarbee, 2000). Through the downslope movement of water, freshwater ecosystems are intrinsically linked with their catchments (Hornung and Reynolds 1995). Lakes continuously accumulate sediments at a relatively high rate and the movement of organic matter, nutrients, pollutants, chemicals and sediment have direct effects on the biota diversity in the lake sediment (Smol, 1992). The changes in water residence time have the most significant influence on the availability of nutrients that will in turn, affect microbial community structure and function (Battarbee, 2000).

Microbial communities often play major roles in facilitating biogeochemical processes lake sediments as the microbial communities respond to physical, chemical and biological changes in their environment (Humayoun *et al*., 2003). Microbial communities that are buried in the lake sediments may also serve as a recorder of past climate and environmental forcing. Hence, this thesis will investigate the potential of microbial communities as indicators of past climate and environmental changes in lake sediments deposited in Lake Suigetsu, Japan.

1.2. Sediment Core

Lake systems have high sedimentation rates (Xu and Jaffé, 2008) and their sediments may preserve a diverse and detailed record of environmental change, including climate for several reasons. First, the flora and fauna in lakes can provide evidence of both local and regional ecological changes. Second, the characteristics of the sediments can provide indications of former environmental conditions. Third, the fossil lacustrine sediments and associated shoreline features often reveal a record of

fluctuations in lake levels in response to climatic changes during the latter part of the Quaternary (Lowe and Walker, 1997). The Quaternary period is defined as the most recent major period of the geological record which includes the Pleistocene (which ended around 10 ka Before Present (BP)) and the Holocene ('wholly recent'; the present warm interval within which we live) (Lowe and Walker, 1997). Additionally, lakes sediments are also suitable for past climate change reconstruction especially in meromictic lakes (chemically stratified lakes with incomplete circulation; Hakala, 2004) where sediments can accumulate quite rapidly, often >1 mm per annum, and can be sub-sampled at high resolution to provide records of historical contamination and past environmental changes (decadal or sub-decadal resolution) (O'Sullivan, 1983).

Lake sediments constitute a minor but important fraction of organic matter, which is essential for a variety of biochemical and geochemical processes (Das *et al*., 2010). Particularly, the benthic animals and microorganisms that are dependent on the components of organic matter for nutrition. The primary source of organic matter in lake bottom sediments is particulate detritus of plants that have lived in the water and on the land surrounding a lake. Organic matter in lake sediments covers the spectrum of being predominantly algal in some lakes to being land derived in others. Bacteria and other microorganisms in the water and sediment of lakes rework and degrade aquatic and terrestrially derived organic matter (Meyers and Ishiwatari, 1993).

1.2.1. Climate

The biodiversity in aquatic environments has been reported to change with environmental temperature (Hall *et al*., 2008). In nature, climatic variations take place within a range of periodicities from the seasonal up to millennial time scales, however, in recent years, the exceptional rates of warming observed pose a threat in interrupting the functions of natural ecosystems particularly, where aquatic environments are exposed to numerous pollutant stresses (Woodward *et al*., 2010).

The regional climate including the precipitation regime can affect catchment hydrology and the depth of the lake waters. The evidence of the past variations in lake levels is often preserved in the lake sedimentary record (Lowe and Walker, 1997). For example, lower levels of lake water may be indicated by the occurrence of horizons of semi-terrestrial or terrestrial peats interbedded with limnic deposits, as well as increased

oxidation or disturbance of lake sediments (Digerfeldt, 1988). Lake level fluctuations can be broadly synchronous at the regional scale, suggesting a climatic control might reasonably be inferred, and hence the lake sediment records can be used as a basis for palaeoclimatic reconstruction (Harrison and Digerfeldt, 1993).

There is evidence for the impact of climate variation on lake sediments. For example, during the Late-glacial period, in the mid- to late Allerød (*ca*. 12-11 ka BP), lake levels were found to be lower throughout Europe, including the Netherlands, northern Germany and southern Sweden, similar to the lake levels recorded towards the end of the Younger Dryas cold phase (between 11 and 10.3 ka BP) (Bohncke *et al*., 1988). These observations phenomena suggest that condition during these periods is significantly drier, over an extensive area of northwest Europe. Similarly, based on the lake level records from the Mediterranean, the conditions during the early Holocene (post Younger Dryas cold phase) suggest a gradual change towards a wetter condition (Lowe and Walker, 1997).

1.2.1.1. Glacial cycles

The glacial-interglacial cycles of the last Quaternary period lasted about 100,000 years (Petit *et al*., 1999). The stable isotope variations from calcareous lake sediments have shown that lake sediments have a direct relationship with palaeotemperatures. According to the oxygen isotope variations obtained from Lateglacial lake sediments in Switzerland, the temperatures of the last glacial-interglacial transition in the Bølling periods (13-12.5 ka BP) are the highest (Lowe and Walker, 1997). Subsequently, during the Allerød periods (12-11 ka BP), gradual cooling with trivial climatic oscillations occurred, and during the Younger Dryas temperatures became very low. This climatic reconstruction is remarkably similar to that based upon beetle data obtained from Lateglacial records (Lowe and Walker, 1997). In this case, lake sediments can therefore offer palaeoenvironmental information based on the analysis of stable isotope ratios in the lake sediments (Siegenthaler and Eicher, 1986; Hammarlund and Lemdahl, 1994).

1.2.1.2. Recent climate change and future predictions

The Earth's climate has already warmed by around 0.6°C over the past century with two main periods of warming, between 1910 and 1945 then from 1976 onwards (Jones *et al*., 1999; McCarty, 2001). Recent studies showed that it is possible to detect

the effects of a changing climate on ecological systems. This suggests that global change may be a current and future conservation threat (McCarty, 2001) to the species and ecosystems. In addition, Walther *et al*. (2002) indicated that irregularity in the warming in various regions can definitely contribute to the heterogeneity in ecological dynamics across systems. Furthermore, changes in precipitation have also occurred. Daily temperature variations have decreased owing to the fact that minimum temperatures are increasing at about twice the rate of maximum temperatures (Walther *et al*., 2002). In most mid- and high-latitude regions, the freeze-free periods continue to lengthen and satellite data have revealed that snow cover and ice extent have decreased about 10% since the late 1960s (Walther *et al*., 2002). Besides, a decadal increment of 0.5 to 1% of precipitations has also been reported in the mid- and high latitudes of the Northern Hemisphere in which it mainly occurs in the autumn and winter. In the sub-tropics, precipitation on the other hand decreases generally by approximately 0.3% every decade (Lowe and Walker, 1997). Similarly, the study by Easterling *et al*. (1997) also showed that in the Northern Hemisphere, winter minimum temperatures between year 1950 and 1993 increased nearly 3°C and 1.4°C for spring maximum temperatures.

In tandem with the apparent global climatic variations in recent decades, other levels of ecological organisation are also affected, such as the shifts in geographic range, changes in species composition of communities and changes in structure and functioning of ecosystems (McCarty, 2001). Although these ecological effects can be allied to recent population declines and species extinctions, both locally and globally, it is not yet possible to convincingly prove that climate change is the cause of these ecological effects and these findings have important implications for conservation biology. Nevertheless, based on available evidence, the changes in the Earth's climate are likely to continue or even speed up over the next 50 to 100 years (Lowe and Walker, 1997). The studies and accurate predictions of how species and ecosystems respond to climatic change have been suggested to be relatively important in the preparation for future conservation challenges (McCarty, 2001). Predictions can be achieved using ecological models in which they must be evaluated by simulating recent ecological changes the same way as the climate models that have been evaluated (Alward, 1999; Pounds *et al*., 1999). This will be especially true for species dependent on unusual and localised combinations of climatic conditions (McCarty, 2001).

1.2.2. Physical and chemical environment of lake systems

The characteristics of the lake sediment can offer information in relation to historical environmental conditions. This is because the variations in the physical and chemical properties reflect the developments in the lake ecosystems as well as changes in the rates at which processes operated around the lake catchment (Lowe and Walker, 1997). The physical conditions of lakes can undergo major seasonal changes which can affect the chemical and biological dynamics of the lake. The morphological and hydrological characteristics of the lake determine whether the responses of the ecosystem are recorded in sediments (Catalan *et al*., 2002). Several factors that will lead to the variations of lake systems are discussed as follow.

1.2.2.1. Lake types

Lakes can possess several origins and their origin is usually related to a catastrophic event such as glacial activity, volcano eruption, or an earthquake (Golterman *et al*., 2011). Tectonic lakes form in area of subsidence caused by folding or faulting and include some of the largest lakes in the world, including Lake Baikal (Siberia) (Todd and Mackay, 2003), Lake Tanganyika (East Africa) (Cohen *et al*., 1993) and Lake Biwa (Japan) (Takemura, 1990). Volcanic origin lakes often formed in extinct craters and calderas (the collapse of the mouth of the volcano, formed after a major eruption) (Golterman *et al*., 2011), and the length of the sedimentary record depends upon the time when volcanic activity terminated. The volcanic lakes in Europe including French Massif Central, central Italy, and Greece, contain sediments which encompass several glacial-intergalcial cycles (Lowe and Walker, 1997).

Glaciers or glacial activity may form two main types of ‘glacial lakes’. These include kettle lakes (develop in hollows created by the melting of buried ice) and lakes dammed behind or between glacial landforms (terminal moraines), resulting from the blocking of drainage outlets (Golterman *et al*., 2011). These sites often contain records that are extended back to the last interglacial or beyond. These lake sequences are important as they provide a continuous record of ecosystems changes, the processes of lake sediment and climate, as well as several glacial-interglacial cycles. These records can therefore offer a means of correlation between terrestrial records, deep-ocean sequences and those from the ice cores. Despite the use of these sequences in stratigraphy subdivision, the palaeoenvironmental significance of these deposits usually

lies more in the fossils these lakes contain than in the nature of the sediments themselves.

1.2.2.2. Temperature

Lakes are primarily linked to climate through ambient temperature, solar radiation, wind (Hostetler and Bartlein, 1990), rainfall directly on the lake and terrestrial runoff (Kilham *et al*., 1996). The changes of stratification and timing of ice formation and loss are driven by wind energy and solar inputs, which will affect mixing depth, the availability of light and nutrient redistributions from direct rainfall and runoff (Kilham *et al*., 1996). Climate change and fluctuations of weather can leave traces on the sediment record by modifying the habitats within the lake (Catalan *et al*., 2002), such as the effect of higher temperatures on the physical properties of the lake water (Jeppesen *et al*., 2009). During warmer periods (i.e. summer), sunlight increases the temperature of lake surface water but at greater depths water is less warmed. Such phenomena will result in the stratification of lake waters into three main layers: the epilimnion (warm surface layer), thermocline (below the level of wind mixing) and hypolimnion (cool bottom water) (Hairston and Fussmann, 2002).

The vertical thermal density gradient in this case can affect the energy required to mix nutrient-depleted surface waters with the nutrient-rich deep waters during seasonal turnover (Winder *et al*., 2009). During warm climate, the upward nutrients flux will be suppressed as the density gradient increases between the upper and lower water layers (Verburg *et al*., 2003; Behrenfeld *et al*., 2006). Besides, higher temperatures can also enhance the depletion in oxygen capacity in the stratified lake waters below the thermocline due to the increase in metabolism (Blenckner *et al*., 2007; Søndergaard *et al*., 2003) and consequently will result in the release of resources (i.e. phosphorus) from the sediment (Catalan *et al*., 2002). Temperature however increases very little in the hypolimnion compared to the epilimnion with warming (Jeppesen *et al*., 2009).

1.2.2.3. Chemistry

Besides temperature, other factors such as pH, nutrients and salinity influence the lakes ecosystem at a local to regional scale (Kingston and Birks, 1990; Schindler *et al*., 1996). Nutrient sources such as carbon, nitrogen or phosphorus are the major elements in most lakes systems (Catalan *et al*., 2002). The availability of carbon,

nitrogen or phosphorus can potentially limit the production of primary producers. Carbon is in effect the most abundant in lakes (CO_2, HCO_3^-, or CO_3^{2-}), followed by nitrogen (NO_3^-, NH_4^+, N_2) and phosphorus (PO_4^{3-}) (Hairston and Fussmann, 2002). Carbon and nitrogen can easily enter lakes by exchange of CO_2 and N_2 from the atmosphere. Nitrogen is usually available in higher concentrations than phosphorus in lakes (Winder *et al*., 2009). Nitrogen is essential for protein synthesis of algal cells. In lakes, nitrogen exists in two major forms; NH_4^+ (ammonia) from the decomposition of organic matter (Knowles, 1982; Tyler *et al*., 2010) or NO_3^- (nitrate) from bacterial denitrification of NH_4^+ (Saunders and Kalff, 2001; Sørensen, 1978). Atmospheric nitrogen (N_2) can also diffuse into the lake water and through nitrogen fixation process, N_2 will be converted to NH_4^+ (Knowles, 1982).

Phosphorus on the other hand, is generally scarce in bio-accessible forms in lakes (Hairston and Fussmann, 2002). Phosphorus is an essential element in nucleic acids, phospholipids and triphosphate (ATP) (Hairston and Fussmann, 2002). The natural source of phosphorus is simply available through the weathering from the watershed of PO_4^{3-} (phosphate) ions, which dissolve rather poorly in water. The concentrations of phosphorus in bio-accessible forms are extremely low in freshwater and are usually utilised very quickly by phytoplankton (Caraco *et al*., 1990; Gunnars and Blomqvist 1997). In addition, under aerobic conditions, PO_4^{3-} can combine with iron to form insoluble salts and sinks to the bottom of the lake, thus will further cause phosphorus to be less accessible to the primary producers such as algae. In the hypolimnion where the condition is anoxic, PO_4^{3-} becomes soluble and during mixing processes, algal growth will be stimulated by PO_4^{3-}. Phosphorus is an important element in lakes, it has been shown to have close relationship to algae production in lakes worldwide (Schindler, 1978) as well as polluted lakes with human waste and laundry detergents (Edmondson, 1991).

1.2.2.4. Salinity

Salt enters aquatic systems from groundwater and terrestrial material via the weathering of rocks or from the atmosphere, transported by wind and rain (Baldwin, 1996; Williams, 1987). Besides, seawater can also infiltrate into nearby freshwater basins during high tide. The relative contributions of these resources depend on factors such as distance inland, climate and geology (Williams, 1987). Lakes with different

salinity, i.e. freshwater and saline lakes have different water chemistry. Salinity can lead to changes in the physical environment that will affect ecosystem processes. For instance, freshwater lakes are usually limited in phosphorus resources, while temperate coastal seas are commonly limited in nitrogen macronutrients (Schindler 1977). According to Nielsen *et al*. (2003), the changes in salinity of freshwater body can potentially change both the penetration of light and the mixing properties, which in turn impact the cycling of nutrients and energy. This is because salt induces aggregation and flocculation of suspended matters, which will remove particles from the water column, hence increases the penetration of light and photosynthesis. As a result, blooms of cyanobacteria are likely to be significant when lake water clarity increases (Donnelly *et al*., 1997). Besides, flocculation may also remove trace elements and nutrients from the water column which are essential for pelagic organisms (Donnelly *et al*., 1997).

Furthermore, saline water incursions into freshwater systems can result in the stratification of lake water into different salinity gradients, such as the meromictic lakes. One of the factors forming of meromictic lakes are the brackish or saltwater incursion into the freshwater basin which receives occasional brackish water pulses and leading to the separation of denser saltwater layers from freshwater (Hakala, 2004). Salinity gradients can reduce the mixing and solute transport within the water column, as well as the movement of oxygen from the surface water to the lake bottom (Legovic *et al*., 1991), which will produce different composition of species at different water layers. Likewise, the nutrients cycling facilitated by the microbial community in the oxic and anoxic layer will be different as well. Apart from oxygen level, the relative proportions of cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and anions (Cl^-, SO_4^{2-}, HCO_3^-/CO_3^-) at the saline layers can also affect the response of freshwater biota to salinity (Radke *et al*., 2002). Bayly (1969) suggested that the ratio of cations Na^+, K^+ to Mg^{2+}, Ca^{2+} is the determining factor of toxicity as monovalent ions are more toxic than the divalent ions.

Often, the incursion of saline water will also result in elevated levels of sulphate, dissolved iron and nitrate. The reduction of sulphate to hydrogen sulphide, a reducing agent, can facilitate the dissolving of iron minerals to release phosphorus (Boström *et al*., 1988) as it has been suggested that sulphide can displace P from insoluble iron Fe^{2+} (Roden and Edmonds, 1997). The study by Blomqvist *et al*. (2004) also indicated that high sulphate content can inhibit nitrogen fixation, therefore promoting nitrogen

limitations in seawater, in contrast to freshwater lakes which are normally limited in phosphorus.

1.2.2.5. Acidity

Acidification of lakes is a common phenomenon in many parts of Europe and North America (Battarbee, 1984; Doka *et al*., 2003). Acid lakes are mostly oligotrophic with slow sedimentation rates, often < 1mm per annum (Battarbee, 1984). Acidity of lakes can be due to the ongoing deposition of compounds such as sulphur and nitrogen oxides, especially in the lakes with poor acid-neutralising capacity (Doka *et al*., 2003). It has been reported that the acidification of lakes can be due to catchment soil acidification and/or acid deposition (Jones *et al*., 1986). The quantity of raw humus from land-use change in lake catchments has also been suggested to lead to the development of acidic lakes (Krug and Frink, 1983). Besides, numerous studies have reported that acidification can lead to reduced species richness across many trophic levels (Walseng and Schartau, 2001) and acidic precipitation and pollution are suggested to be one of the main threats to biodiversity at the population and ecoregion level in Canada and the U.K (Doka *et al*., 2003). Battarbee (1984) reported that the first and foremost sign of acidification is the loss of planktonic diatoms when pH values fall between 5.5 and 5.8. Acidification below pH 5.5 can further lead to the reduction of species typical of nearly neutral water and when pH drops to 4.5, these taxa will be replaced by taxa that are acidophilic.

1.2.3. Means of analysing change

Biological evidence, in the form of plant and animal remains, has always been the foundation in the reconstruction of Quaternary environments (Rymer, 1978). The analysis of fossil evidence employs uniformitarian principles, namely that a knowledge of the factors that influence the abundance and distribution of contemporary organisms enables inferences to be made about environmental controls on plant and animal populations in the past (Lowe and Walker, 1997). Sediments often contain surprisingly abundant and diverse fossil components and the fossil evidence upon which Quaternary palaeoecological studies are based mainly falls into two major categories; the macrofossil and microfossil evidence. Macrofossil evidence usually can be identified by eye or with low power magnification (up to *c*. × 40), which includes whole skeletons of large vertebrates or small fragments of plant or animal remains. Microfossil evidence on

the other hand consists of remains of former biota that can only be identified by using microscopes (Lowe and Walker, 1997). Fossil survivals can be best preserved particularly in anaerobic environments where deterioration, resulting from the activities of microorganisms and chemical activity is minimal, rather than those where oxidation can occur (Allison and Briggs, 1991). Pollen grains, diatom and plant macrofossil are analyses that are widely used as means of analysing environmental and climate change. These analyses are important as they can be comparable to the analysis of microbial community structure and function in the sediment records.

1.2.3.1. Pollen analysis

Microfossils are generally less than 1mm in size which includes pollen, diatoms, algae, fungal spores and zooplankton. Pollen stratigraphy is the most useful technique of all in the reconstruction of Quaternary climate as each pollen part is unique to the plant from which it came (Lowe and Walker, 1997). This technique was first employed in the 1920s for the correlation of Quaternary stratigraphy units, aiming to reconstruct vegetational history (Delcourt and Delcourt, 1991), to investigate the impact of human activities on late Quaternary vegetation as well as the landscape in various parts of the world (Behre, 1986). Pollen grains and spores are frequently dispersed in very large quantities to maximise the opportunities of successful pollination or gametophyte growth and they usually accumulate on the ground surface or in water bodies. Those that have been incorporated and preserved in the sediments will be utilised for pollen analysis. Pollen grains and spores are usually well preserved in lake and pond sediments and in peats. The degree of pollen grain preservation is dependent upon the environmental condition, whether or not it is anaerobic and the thickness and structure of the outer layer of pollen grains (exine). Exine serves as a resistant wall that protects the young gametophyte from desiccation and microbial attack, therefore can be preserved in the sediments (Lowe and Walker, 1997).

Pollen response surfaces are considered a powerful new tool for climatic reconstruction and are being increasingly widely used in the development of integrated models of past global climates, such as the reconstruction of Quaternary climates (Lowe and Walker, 1997). These and subsequent approaches have relied largely on indicator species in the pollen records, such as plants with known climatic affinities (Zagwijin, 1994). In addition, pollen records from lake cores also enable inferences to be made

about the history of a particular lake ecosystem (Charman, 1994) while pollen diagrams (3D pollen analysis) enable the mapping of the changes of the local vegetation cover over time (Smith and Cloutman, 1988). In coastal regions, pollen analysis can help to elucidate the history of sea-level change, through the changes between saltmarsh and terrestrial or freshwater plant communities (Shennan *et al*., 1994).

1.2.3.2. Diatoms

Diatom analysis is extremely useful as an indicator of local habitat changes, specifically in lake sediments and in both shallow and deep marine deposits (Battarbee, 2000). Diatoms are microscopic, unicellular members of the Bacillariophyta of the algal kingdom. They can be found in a wide range of aqueous to sub-aqueous environments and they make up approximately 80% of the world's primary producers (Lowe and Walker, 1997). Environmental parameters such as water acidity and salinity, oxygen availability, nutrient content and water temperature are the important variables, responsible for the distribution of diatom species (Battarbee, 1984). Freshwater diatoms, for instance are largely controlled by salinity, pH and trophic status, while sea-surface temperatures, oceanic frontal contrasts and nutrient up-welling influence the distribution of many marine taxa (Villareal *et al*., 1993). Changes in any of these parameters can have a major effect on the structure and composition of the diatom community. However, the interpretation of diatom assemblages can be difficult at times due to the fact that diatom valves are light and can be easily transported. Therefore, difficulties arise when there is frequently a complex mixture of marine, brackish and freshwater forms of diatoms found in the estuarine sediments, while lake muds may contain diatoms derivatives not only from the lake ecosystem itself, but also from inflowing streams and catchment soils (Lowe and Walker, 1997).

1.2.3.3. Plant macrofossils

The analysis of plant macrofossils not only can provide valuable complementary information to microfossil data, but also can provide an independent approach to the reconstruction of environmental conditions (Birks *et al*., 2005). Plant macrofossils are the remains of vascular plants including fruits, seeds, stamens, buds and scales. In archaeological contexts, carbonised plant macrofossils are encountered, mainly in the form of wood or seeds (Lowe and Walker, 1997). Plant macrofossils can be found in a variety of depositional environments and they are most commonly found in lacustrine

and fluviatile sediments (i.e. fine alluvium) as well as acid peats. Specifically in the acid peat deposits, the remains are best preserved as fossils can be protected from oxidation. In addition, among the fossil remains, fruits and seeds can survive in most deposits and they are resistant to decay, hence can adapt and withstand periods of dormancy. However, seeds of grasses are rarely found in fossil form (Birks and Birks, 1980). The leaves of deciduous trees, which have delicate structure, are rarely preserved in lake sediments except as very small fragments due to the fact that they are highly vulnerable to mechanical breakdown and decomposition. On the other hand, fossil of lower plants such as mosses are better preserved. Therefore, in general, Quaternary macrofossil analysis focusses primarily on the study of wood, seeds, fruits and mosses, which can enhance the information provided by a limited number of easily identifiable plant remains such as conifer needles and certain leaves. In terms of interpretation, plant macrofossils data are quite limited in providing valuable information especially in the reconstruction of regional vegetational patterns. This refers to the lack of data in relation to the composition of former plant communities that have been growing in and around the deposition site. A number of limitations are also associated with the analysis of using plant macrofossils. This often includes an inadequate quantity of remains in sediments which is difficult to compare to pollen grains. Even so, plant macrofossil evidence has proved useful to aide pollen analysis in differentiation between plant types when pollen grains are almost identical (Birks *et al*., 2005).

1.2.4. Dating methods

Dating methods enable the age of fossils, sediments or rocks to be established directly in years BP. There are several dating techniques of fossil records in the Quaternary time range that basically fall into three broad categories; i) methods that provide age, ii) methods that establish age-equivalence and iii) relative age methods (Lowe and Walker, 1997). However, many of the dating techniques currently employed in Quaternary research can only be applied to restricted spans of Quaternary time and those that are widely used are discussed as follow.

1.2.4.1. Radiocarbon dating

Radiocarbon dating (or simply carbon dating) is one of the radiometric techniques that are based on the radioactive properties of certain unstable isotopes which undergo spontaneous changes in atomic organisation in order to achieve a more

stable atomic form (Cronin, 2010). One of the widely used radiocarbon isotopes is the decay of carbon-14 (^{14}C), to estimate the age of organic materials, such as wood and leather, up to approximately 58,000 to 62,000 years BP (Lowe and Walker, 1997). ^{14}C atoms are rapidly oxidised to carbon dioxide and throughout the atmosphere, they become mixed with other molecules of carbon dioxide ($^{12}CO_2$) and subsequently are taken up by the oceans and living organisms. In other words, ^{14}C that is continually produced in the upper atmosphere will be stored in various biosphere and hydrosphere worldwide. Radiocarbon dating is based on four fundamental assumptions; 1) that the production of ^{14}C is constant over time; 2) that the ^{14}C: ^{12}C ratio in the biosphere and hydrosphere is in equilibrium with the atmospheric ratio; 3) that the decay rate of ^{14}C can be established and 4) that a closed system has existed since the death of the organism (Walker, 2005). According to Lowe and Walker (1997), all living matter absorbs carbon dioxide during the building of tissues and in a ratio that is broadly in equilibrium with atmospheric carbon dioxide. Upon death, ^{14}C within the organic tissues will continue to decay, without any replacement taking place. Therefore, if the rate of decay of ^{14}C is known, the date of death can be calculated by measuring the remaining ^{14}C activity. The activity of the sample in this case is interpreted as indicating 'age', based on a number of assumptions. However, based on the radiocarbon assays on fossil corals, true age can be underestimated by 2500 at 16,000 BP and 3500 at 20,000 BP on a longer time course (Bard *et al*., 1990), due to the fluctuations in deep ocean ventilation (Broecker *et al*., 1990). Therefore, radiocarbon timescale should be calibrated against chronology of calendar years, by comparing ^{14}C dates with those obtained from the same samples of material using independent dating methods (Cronin, 2010).

1.2.4.2. Lead-210 (^{210}Pb)

^{210}Pb is one of the daughter nuclides from the radioactive decay of radon gas (^{222}Rn). It is a naturally occurring but unstable isotope, with a half-life of 22.26 years (Battarbee, 1984). ^{210}Pb can be accumulated in soils, peats, glacier ice, lacustrine and marine sediments, and subsequently decays to a stable form of ^{206}Pb within the interval of *c*. 150 years or so (Walker, 2005). To establish the rate of sedimentation, dates are usually calculated assuming a constant rate of supply of unsupported ^{210}Pb to the sediment through time (Lowe and Walker, 1997). However, a major issue encountered for this technique is that most sediments contain only small amounts of ^{210}Pb that is

derived from the uranium-series decay chain, thereby the 'supported' ^{210}Pb determined should be subtracted from the 'unsupported' ^{210}Pb produced in the atmosphere (Cronin, 2010). This dating technique is often utilised in limnological studies, for instance, lake sedimentation rate (Appleby and Oldfield, 1983), laminated lake sediments (Appleby *et al*., 1979) and human impact on lake ecosystems (Varvas and Punning, 1993). Besides, ^{210}Pb dating has also been applied in dating peats and alpine glacier cores, as well as polar ice sheets (El-Daoushy, 1986).

1.2.4.3. Tephrachronology (Volcanic layers)

The ash or tephra after a volcanic eruption often spreads rapidly over a relatively wide area in which it forms a thin layer over concurrent peat surfaces, floor of lakes, estuarine sediments, river terraces and even in deep-sea sediments (Lowe and Walker, 1997). Ash layers stand out quite distinctively among the sedimentary sequences in cores, usually in light-coloured horizons which can be easily identified by several methods, including granulometric characteristics, petrographical and mineralogical properties and geochemical signatures (Einarsson, 1986). These techniques not only can be used to distinguish between ashes, they may also enable source to be established such as the identification of primary mineral constituents. Besides, dated tephra can also serve as the stratigraphical position in relation to dated tephra layers, palaeomagnetic correlations, annually laminated sediments, biostratigraphical methods (e.g. pollen analysis) and relationships to oxygen isotope stage boundaries in deep ocean sediments (Einarsson, 1986). Tephrochronology has now been employed in many areas and it has been shown to have considerable potential as a technique, both as correlative tool and in the development of local and regional chronologies. However, it is simply applicable for regionally relative dating only if the individual ash layers can be spatially limited by several factors, such as the magnitude, type of volcanic explosion, the strength and direction of prevailing winds and the particle size of tephra (Walker, 2005).

1.2.4.4. Varve chronology

Periodical accumulations of sediments, forming bands of laminae, composing of sine sand, silts or clay, are quite common in the geological records. Such sediments are usually referred to as rhythmites. If these layers can be shown to be annual variations in the supply of sediment they are termed varves (Lowe and Walker, 1997). The word 'varve' is from a Swedish word for 'layer'. In 1912, annual laminations were first

described as varves by DeGeer to illustrate proglacial varves (DeGeer, 1912). 'Varve' was formerly used solely to describe proglacial varves for the verification of time elapsed since the Last Glacial Termination. However, since the late 1970s and 1980s, 'varve' is used to describe all types of annually laminated sediments regardless of their environments, not only for proglacial sediments but also non-glacial ones (Mackay *et al*., 2003).

Varves are common in many temperate areas apart from proglacial lakes. Varve sedimentation as well as biomass production are mainly affected by seasonal variations in lakes and in certain temperate lakes, chemical precipitation also varies with season (Lowe and Walker, 1997). The formation of sediments largely depends on the condition of the lake bottom and in the anoxic lake bottom, the numbers of bottom-dwelling fauna are restricted, often with no vertical water circulation extending to the lake bottom, therefore fine laminations can be preserved (Ojala *et al*., 2012). Seasonal variations can also result in the accumulation of organic detritus which will contribute to organic varves at the bottom of the lake waters. For instance, the precipitation of $CaCO_3$ will lead to the development of light summer layers, while dark winter layers are formed by rich organic humus (Peglar *et al*., 1984; Rapp and Hill, 2006). Diatom blooms during spring and early summer can also contribute to annual laminations in lake sediments which have been observed in both interglacial (Turner, 1975) and more recent sequences (Simola *et al*., 1981).

The varved sediment that does not undergo mixing despite seasonal rates of sediment increments has several advantages for palaeoenvironmental research as it can potentially provide information on past environmental changes by examining variations in structure, composition and thickness of the seasonal laminae (Ojala and Alenius, 2005). Nevertheless, the most important feature of varved sequences are that they provide a natural and continuous timescale which is known as the varve chronology for palaeoenvironmental studies which can be evaluated and calibrated with other dating techniques (Ojala *et al*., 2012).

Varve chronologies have been applied to determine the patterns of regional deglaciation, the duration of Quaternary time periods (Björck *et al*., 1987; Goslar *et al*., 1993; Lotter, 1991) as well as to calibrate the ^{14}C timescale (Stuiver, 1971). In fact, varve chronologies can be considered as a complete representation of a calendar-year

timescale (Ojala *et al*., 2012). The laminated surface sediments are the present-day deposits while the deeper varved sediments represent past environments. However, adverse weather conditions in particular years can reduce the input of sediment, biomass and even chemical precipitation into lake sediment which will result in the formation of either thin layer of varve or none (Lowe and Walker, 1997). In this case, incorrect estimation of age can result. Furthermore, events such as flood may also complicate the interpretation of varve record particularly in lacustrine environments as flood events are essentially the non-annual nature of lacustrine sedimentary sequences (Leonard, 1986). Crossdating of varves using tree-ring chronology for example is therefore essential to minimise the issues of false or missing varves.

1.3. Microbial ecology approaches

Since the 1940s, the importance of microorganisms to biogeochemical processes in aquatic biospheres has been recognised as microorganisms were identified to play important roles not only in the food webs, sediment functioning, but also numerous biogeochemical cycles, including the recycling of organic compounds, plant productivity and climate regulations (Gugliandolo *et al*., 2011; Kondo *et al*., 2009; Schauer *et al*., 2000; Smalla *et al*., 2007). Studies of microbial ecology in such environments have become a main focus in recent years due to the fact that lakes and other aquatic ecosystems have a more significant role in the global carbon budget than previously acknowledged (Comte *et al*., 2006).

Methods that can be used to measure microbial ecology from lake sediments can be classified into two groups: biochemical-based and molecular-based approaches. Biochemical-based analyses include pigments (Ohkouchi *et al*., 2005) and lipid biomarkers (Dong *et al*., 2006; Jiang *et al*., 2007) which have been applied to assess the microbial biomass and the community structure in saline meromictic lakes. In particular, lipid markers have been largely utilised in the study of sedimentary environments owing to the fact that membrane lipids are essential components of every living cell that are structurally diverse and biologically high in specificity (Boschker and Middleburg, 2002; Parkes 1987).

Phospholipid fatty acid (PLFA) analysis is one of the lipid biomarkers that is known to be useful for investigating microbial community structure, evaluating biomass

and physiological changes in environmental samples (Dong *et al*., 2006). This analysis can be applied on pure cultures or directly to environmental samples including soils and sediments. This method relies on the basis that lipids are considerably different between microbial groups, which thus can be distinguished from each other (Boschker and Middleburg, 2002; Dong *et al*., 2006; Zelles, 1999). The advantage of using this approach is that it can detect rapid changes in microbial community structure through the changes of PLFA patterns. However, the classification of microorganisms using PLFA profiles is rather difficult due to the lack of standardised techniques, qualitative and quantitative information on the distribution of fatty acids across microbial species. This method is also less accurate as PLFA patterns can be overlapping between individual species. In addition, PLFA analysis is only valid to metabolically active populations but not dead cells and only certain PLFA that are present can be extracted from soil (Zelles, 1999).

Molecular techniques have been increasingly applied in microbial ecology studies, for instance, the hypervariable regions of the highly conserved 16S rRNA gene are usually targeted to analyse the structure and species composition of microbial communities. Such methods were first applied by Muyzer *et al*. (1993) to explore the microbial communities from natural environments. In the recent years, other molecular techniques such as quantitative PCR (Q-PCR) (Jiang *et al*., 2008), terminal restriction fragment length polymorphism analysis (T-RFLP), (Bhattarai *et al*., 2012), denaturing gradient gel electrophoresis (DGGE) (Koizumi *et al*., 2004; Lim *et al*., 2011), clone libraries (Humayoun *et al*., 2003), phylochip (Klepac-Ceraj *et al*., 2012) and metagenomics sequencing (Delmont *et al*., 2011) have also been utilised to identify the microbial communities in lake environments. According to Kim *et al*. (2013), molecular fingerprinting techniques such as DGGE and T-RFLP are the most popular techniques for the investigation of microbial communities from natural environments. In these approaches, amplified PCR fragments of varying gene sequences or lengths are passed through a gel and separated based on their differing mobility. The variation in sequence or length due to the hypervariable regions of 16S rRNA gene, allow the discrimination of different species (Gilbride *et al*., 2006; Muyzer and Smalla, 1998). Although these methods are inexpensive to use, there are biases associated with these approaches. A common drawback of DGGE approach is that the partial 16S rRNA gene sequences do not always allow discrimination between species as different bacteria from the same genus or different genera can yield identical partial sequences (Marzorati *et al*., 2008;

Muyzer and Smalla, 1998; Schütte *et al*., 2008). One way to resolve this limitation is the cloning and sequencing of excised bands so that cleaner sequences can be achieved. However, the analysis of multiple clones from complex communities from the natural environments will be excessively laborious and time-consuming (Díez *et al*., 2001).

Microarray and pyrosequencing represent new powerful methods that have been used to investigate microbial ecology (Delmont *et al*., 2011; Klepac-Ceraj *et al*., 2012). Phylochip (phylogenetic oligonucleotide arrays) is a type of DNA microarray that is designed to detect any microorganisms from any environmental samples based on complementary hybridisation of microbial amplified 16S rRNA gene to labelled oligonucleotide probes (Wagner *et al*., 2007). This technique can simultaneously resolve more than 8,000 bacterial strains, which are two fold greater than molecular finger printing techniques (DeSantis *et al*., 2007). Although the microbial community at low abundance can be detected through Phylochip microarrays, novel microorganisms from environmental samples cannot be recognised, given that specific probes have to be designed beforehand to detect such organisms (Klepac-Ceraj *et al*., 2012). Hence, this technique is only suitable for the identification of known taxa. DNA pyrosequencing technique is based on the principle of DNA synthesis and the detection of light during the released of pyrophosphate. During DNA polymerisation, when deoxyribonucleotide triphosphate (dNTP) is added to the DNA strand, pyrophosphate will be released and converted to ATP by ATP sulfurylase. Nucleotides are added one by one and any unincorporated nucleotides will be degraded by apyrase enzyme before the next nucleotide is added. Subsequently, luciferase uses ATP as energy to oxidise luciferin and produces light. Light is detected as peak by the device and the height of the peak represents the amount of ATP used and hence proportional to the number of dNTPs being incorporated (Ronaghi, 2001). One of the major drawbacks for this technique is that it is currently limited up to 500 bp of fragment size which only enables discrimination at species levels, therefore unsuitable for full 16S rRNA gene analysis (Kumar *et al*., 2011). However, pyrosequencing allows the detection of novel taxa that were previously unknown, unlike DNA microarrays which can only identify known bacterial sequences.

1.4. Field site

1.4.1. Lake Suigetsu

Lake Suigetsu (35°35′N, 135°53′E, 0 m above sea level) (Kossler *et al*., 2011) is located on the coast of Wakasa Bay in southern Fukui Prefecture, in central Honshu, Japan (Figure 1.1) (Kondo *et al*., 2009). It has a diameter of 2 km (Nakagawa *et al*., 2005), an area of 4.3 km^2 (Kitagawa and van der Plicht, 1998a) and a flat basin as deep as *ca*. 34 m (Kitagawa and van der Plicht, 1998a; Kondo *et al*., 2009; Matsuyama and Saijo, 1971; Nakagawa *et al*., 2005).

Lake Suigetsu falls in the tectonic origin system which is known as the 'Mikata Five Lakes' system (Figure 1.1) (Kitagawa and van der Plicht, 1998a). It is one of the study sites that is prominent among the Quaternary science community (Nakagawa *et al*., 2012). It is known to provide an ideal radiocarbon calibration model as the records are derived exclusively from terrestrial plant macrofossils within the varved sediment (Kitagawa and van der Plicht 1998a, 1998b, 2000).

Among the Asian lakes, Lake Suigestsu is one of the most suitable study sites for the chronology of palaeoclimatic shifts that fulfills the objectives of INQUA-INTIMATE (Blockley *et al*., 2012). INQUA-INTIMATE is an international project which aims to unveil the comparative timing of abrupt climate changes from the Lateglacial (60,000 yr BP) to early Holocene (8,000 yr BP) through the use of independent chronologies collectively obtained from the records of terrestrial, marine and ice cores (Lowe *et al*., 2008; Blockley *et al*., 2012).

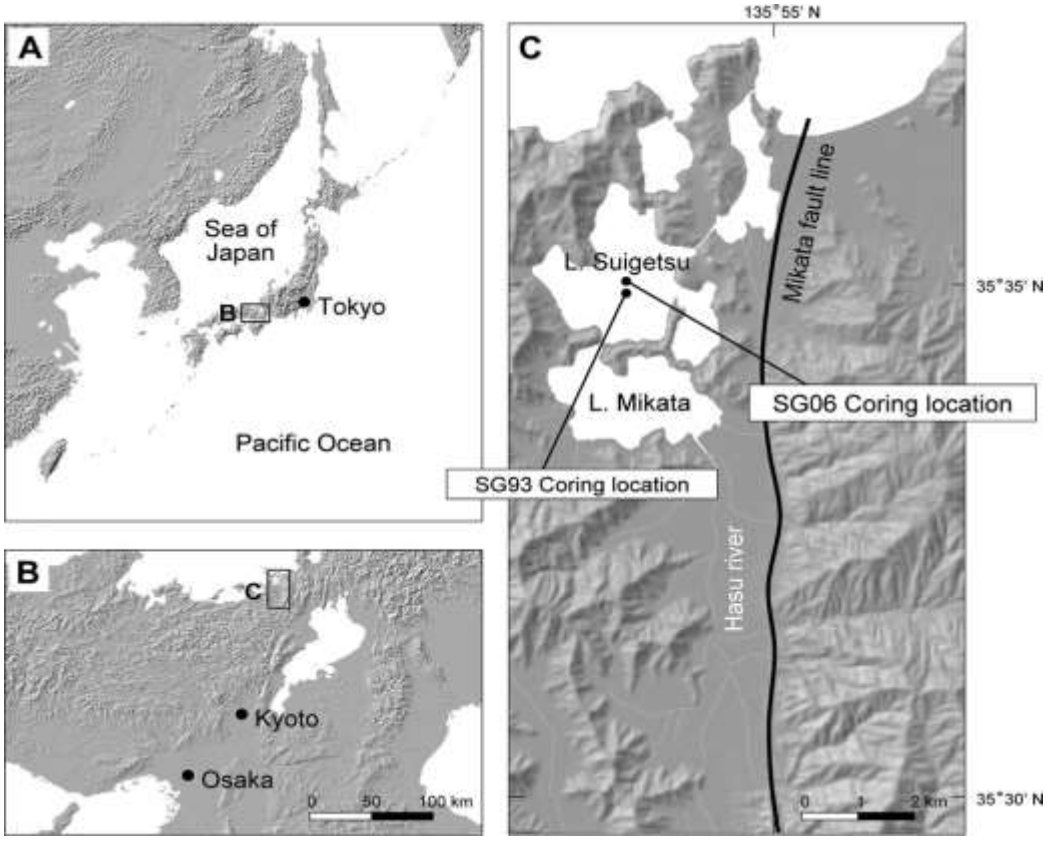


Figure 1.1: Location of Lake Suigetsu (Nakagawa *et al*., 2005; 2012)

1.4.1.1. Meromictic characteristics of Lake Suigetsu

A common feature of meromictic lakes is that there is a lack of water circulation and the lake waters are permanently stratified into two layers which do not interfere with each other (Hakala, 2004). Geographically, Lake Suigetsu is surrounded by lakes and hills (Figure 1.2) (Kossler *et al*., 2011) and a permanent chemocline is formed in Lake Suigetsu, between 3 m to 8 m, separating the aerobic freshwater mixolimnion of the upper water layers from the anaerobic water below which is a saline sulfidogenic monimolimnion (Kondo *et al*., 2000, 2006; Matsuyama, 1973a, 1973b; Matsuyama and Saijo, 1971). Hutchinson (1937) indicated that under certain circumstances, meromictic condition of a lake can result from an external event (i.e. the incursion of saline water), continuous supply of denser mineralised water into the lake or the increase of electrolyte concentration due to highly decomposed organic material at the lake bottom. In addition, the development of meromictic lakes can also be attributable to its location, such as surrounded by thick forest, sheltering landscape or lake morphology which yielded minimal wind action. After an extended period of time, the lake waters will become permanently stratified into different transition zones. Hutchinson (1937) termed the layer separating the upper and lower strata as chemocline and the layer above the

chemocline as mixolimnion while the water layer below the chemocline is characterised as monimolimnion (Hakala, 2004).

The location of Lake Suigetsu has also given rise to the formation of laminated sediment at the bottom of the lake. It has connections to several lakes in close proximity including the freshwater Lake Mikata and the polyhaline Lake Hiruga (Matsuyama and Saijo, 1971). The main water inflow originates from the Hasu River, which first reaches Lake Mikata before Lake Suigetsu. Lake Mikata in this case, serves as a filter, indirectly helping to strain off most of the coarse materials conveyed from the Hasu River (Figure 1.2) (Kawakami *et al*., 1996). Likewise, the hills and steep slopes surrounding Lake Suigetsu which are sheltered by dense forest vegetation also contribute to the fine lamination deposited in Lake Suigetsu as it helps to minimise landslide and soil erosion activity in spite of weather circumstances (Kossler *et al*., 2011).

Figure 1.2: Lake Suigetsu and its surrounding lakes

Due to the lack of the mixing of the lake waters and the deposition of fine materials into the lake, the sediment is less disturbed and a steady depositional environment was established in the lake basin for approximately 100,000 years which ultimately produced a well-defined laminated sediment (varves) (Figure 1.3) (Nagayoshi *et al*., 2007; Kitagawa and van der Plicht, 1998a). The regional climate around Lake Suigetsu can be characterised by both summer and winter monsoons. During summer, Japan receives mostly south-easterly winds and humidity from the Pacific Ocean, while in winter the dominant north-westerly winds come from Siberia. The wind over the relatively warm surface water of the Sea of Japan picks up much moisture and eventually provides heavy winter precipitation to Japan, particularly along the western side of the country, including the Lake Suigetsu region (Nakagawa *et al*., 2012). Therefore, the surrounding temperatures of Lake Suigetsu can represent proxies for the cold of Siberian air mass in winter and the warmth of Pacific air mass during summer monsoon intensity, with related winter and summer precipitations (Nakagawa *et al*., 2006). In addition, due to this strong seasonal shift, varves with various thickness and texture are formed (Mackay *et al*., 2003), which are composed of rich diatoms layers and aeolian dust and humus layers (Nakagawa *et al*., 2005).

Figure 1.3: Fine annually laminated varves of SG06 core of Lake Suigetsu (Nakagawa *et al*., 2012)

1.4.1.2. Internationally recognised importance of the Lake Suigetsu

In order to reconstruct past climate chronology, several records can be used to develop the ^{14}C radiocarbon calibration curves. These include the partially varved sedimentary records from the Cariaco Basin (Hughen *et al*., 2006), uranium series-dated corals (Fairbanks *et al*., 2005) and marine sediment (Reimer and Reimer, 2001). Tree-ring records are one of the archives known to provide nearly accurate calendar age year based on to their annual growth bands (Staff *et al*., 2011). However, there is a limitation associated with this calibration model in that it only extends up to 12,593 calendar years BP (Schaub *et al*., 2008).

The occurrence of varves sediment in Japan was first discovered when four short sediment cores (SG1-4) were obtained from Lake Suigetsu in 1991 and 1993 (Kitagawa and van der Plicht 1998b). Subsequently in year 1998 and 2000, Kitagawa and van der Plicht had generated a radiocarbon profile by combining more than 300 radiocarbon ages measured from the terrestrial leaf macrofossils based on the varved sediment of Lake Suigetsu (Kitagawa and van der Plicht, 1998a). This study revealed the extension back to the radiocarbon detection limit (*ca*. 50,000 year cal BP) and it was an early attempt to produce a radiocarbon dating model beyond the tree-ring limit (11,400 cal BP at that period of time) (Kromer and Becker, 1993).

Lake Suigetsu became internationally recognised when this model was published. However, there were several issues allied with core SG93. It was later recognised as 'young varves' that had been underestimated due to the undercounting of the varves and over-counting of the annual bandings in other sites (van der Plicht *et al*., 2004). In summer 2006, another research team funded by the UK Natural Environment Research Council (NERC) conducted a new sediment coring exercise at the lake's depocenter of 34 m, reaching the base of the sedimentary profile (73.19 m below the lake bottom) (Staff *et al*., 2011). Cores were recovered from four parallel boreholes (A, B, C and D), all within 20 m horizontal distance from each other, with fully overlapping core segments (Figure 1.4) and no chronological gaps in between, unlike core SG93 (Nakagawa *et al*., 2012). Fukusawa (1995) indicated that the annually laminated sedimentary record of Lake Suigetsu is a 'natural timekeeper' and reliable recorder of environmental change, therefore, is capable of providing an independent whilst high

resolution and precise age control of event stratigraphy for palaeoclimatic reconstruction (Nakagawa *et al*., 2003; 2005; 2006).

The recovered overlapping sediments contain finely laminated varves and occasionally thick layers are also found in the sediment cores. These layers are suggested to be the event layers which are categorised into two empirical types; i) the light-coloured massive clay layers and ii) a slightly coarser clay layers, but generally much thicker and with dark coloured layer underlying. It is hypothesised that the light-coloured clay layers were formed by large flood events while thicker layers with coarser material are interpreted as small-scale turbidites. Such turbidites can either be formed by surface runoff of the slopes around the lake or caused by earthquakes (Nakagawa *et al*., 2012).

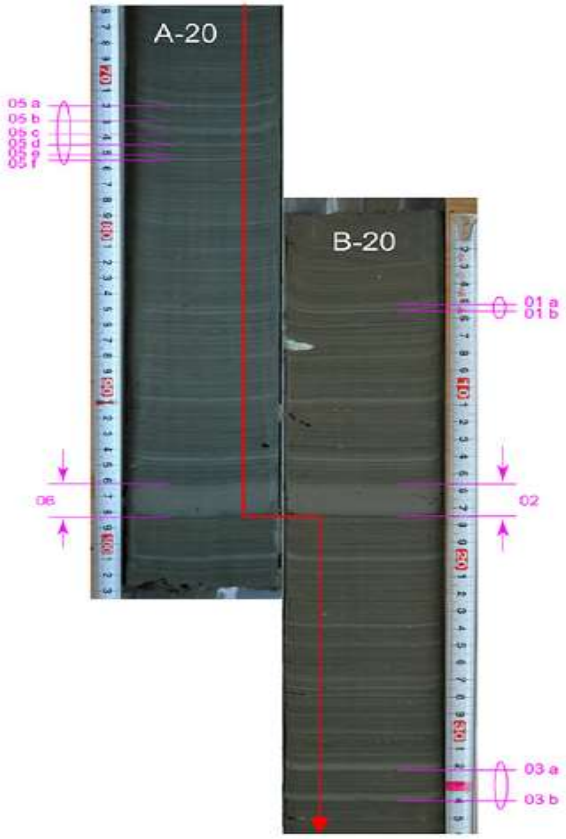


Figure 1.4: Example of overlapping lamina patterns from parallel borehole A and B (Nakagawa *et al*., 2012)

1.4.2. Climate record from Lake Suigetsu

The majority of macrofossil samples in the sediment cores are tree leaves and small twigs, bark, seeds, and a few segments of insects are also found (Staff *et al*., 2011). Sampling undertaken for ^{14}C calibration was throughout the last ~12,000 cal yr, almost entirely representing the Holocene (Nakagawa *et al*., 2003). In addition, based on the pollen analysis, Nakagawa *et al*. (2005) have reconstructed the past climate of the Late Quaternary in central Japan.

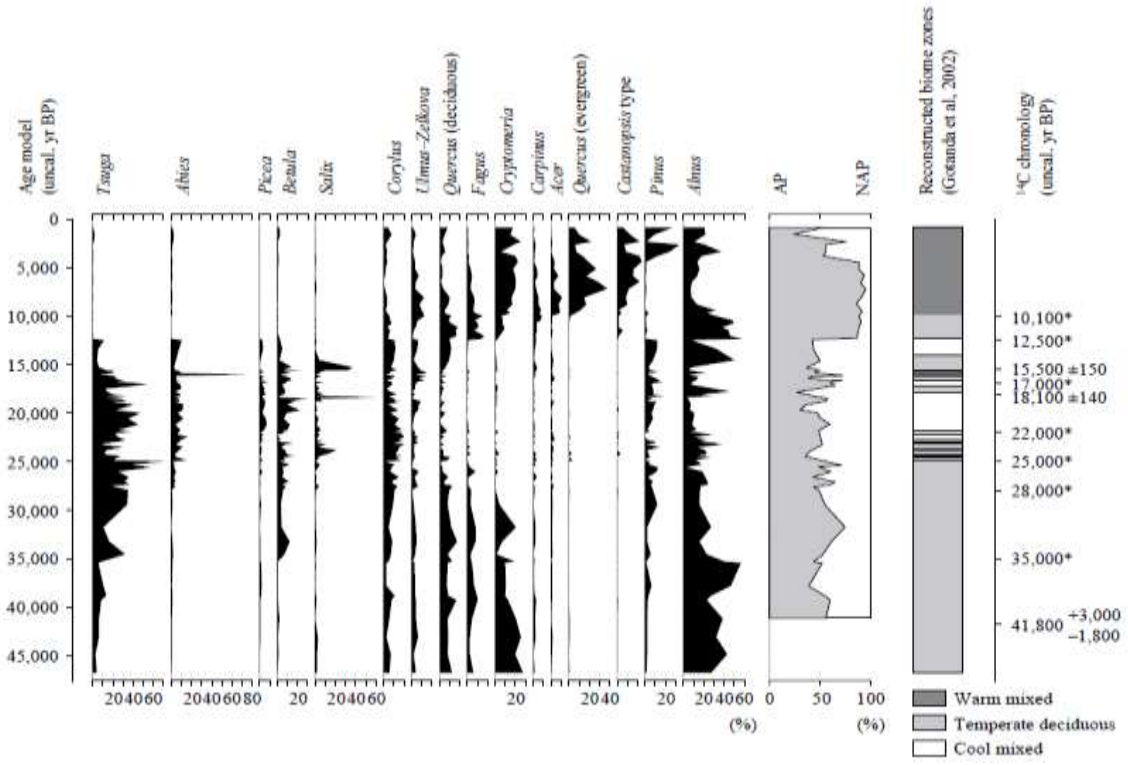


Figure 1.5: Biome reconstructed based on pollen records of Lake Mikata, spanning over the last 45,000 BP (Nakagawa *et al*., 2002).

Based on the SG06 varve chronology, the vegetation around Lake Suigetsu before 15,000 BP was identified as cool mixed forest (COMX) that comprised of coniferous trees and deciduous broad-leaved trees (an example shown in Figure 1.5). During this interval, the mean annual temperature increased from *ca*. 5 to 10°C, suggesting a continuation of deglacial warming which started at about 20,000 BP (*ca*. 3°C) in the region of Lake Suigetsu (Nakagawa *et al*., 2002). Changes in vegetation from COMX to typical temperate deciduous (TEDE) forest of Japan were identified at the boundary of 15,000 BP as coniferous pollen disappeared. Although the change at this transition corresponds to the onset of the GI-1e/Bølling period in the North Atlantic, it is not possible to conclude that the change was as abrupt as the onset of the GI-1e/Bølling phase in the North Atlantic. Furthermore, the climate around Lake

Suigetsu became colder between 12,300 and 11,250 BP. The vegetation showed the increase in *Fagus crenata* and a total absence of the coniferous trees, indicating cooler condition that is comparable to the winter season (Nakagawa *et al*., 2005). During this period, the climate was unstable with pollen productivity frequently changed, and the abrupt increase of *Pinus* subgen. (typical pioneer species in Japanese temperate forest; Nakagawa *et al*., 2005), indicating an abrupt climate change. At the end of the cold phase, the vegetation around Lake Suigetsu was TEDE forest with temperature approximately 5°C lower than the present. The temperature continues to rise to the present level and warm mixed forest (WAMX) around Lake Suigetsu was established sometime after 10,000 BP which is likely to suggest the beginning of the Holocene period.

The reconstruction of a biome based on Lake Suigetsu is in agreement with Lake Mikata's (Gotanda *et al*., 2002), however the study was mainly focused on the period during the Last Termination. Based on the biome reconstructed from the pollen records of Lake Mikata, the period from > 40,000 to 25,000 BP was represented by TEDE forest. During the period from 25,000 to 22,000 BP, the vegetation was classified by both TEDE and COMX forests as the affinity scores of both biomes are close. From the period of 22,000 to 18,000 BP, COMX forest was dominant whereas period between 18,000 to 15,000 BP, the vegetation began interchanging between COMX and TEDE forests.

Chapter 2 Aims and Objectives

The primary aim of this research was first to identify possible molecular biomarkers by elucidating and identifying the diversity of both bacterial and archaeal communities in the sedimentary records of Lake Suigetsu, spanning the past *c*. 150,000 years, by comparing to the biome reconstructed based on Lake Mikata. Specific objectives addressed during the course of this study are outlined as follow;

Chapter 4: Molecular fingerprinting analysis of fossil DNA throughout the 73 m sediment core of Lake Suigetsu (c. 150,000 years) to elucidate bacterial community structure and diversity patterns to search for potential biomarkers for past climate reconstruction.

Chapter 5: Lake Suigetsu sediments have records of a seawater invasion event in 1664 AD. So, molecular analyses of the effects of environmental changes on bacterial community structure and diversity, particularly the salinity effects were targeted. Investigations were focused on bacterial community structure and diversity before and after seawater invasion. The possible timeline and depth at which the salinity influx event was the initial stroke was identified and the taxa between the anoxic saline lake waters and brackish sediments were compared.

Chapter 6: Lake Suigetsu sediments also recorded information on the glacial to postglacial climate condition between 15,000 to 14,500 BP, as indicated by pollen records. Thereby, an exploration of the effects of climate change on bacterial diversity and community structure in sediments from a transition from cooler to warmer climate condition was performed. Identification of whether the bacterial taxa demonstrated a measurable change during these periods as well as if there is strong correlation between specific taxa and climatic conditions was also investigated.

Chapter 7: Culture-dependent analysis to distinguish bacterial diversity in brackish sediment (335 BP) from freshwaters (6,860 BP and 10,911 BP), to allow the determination of whether ancient bacteria from fossil sediments are ‘culturable’. The microbial data between this study and molecular techniques were also compared.

Chapter 8: Polyphasic approach to the characterisation of potentially new *Actinobacteria* strains recovered from the freshwater sediment records and description of novel species of *Dermacoccus*, *Dietzia*, *Leifsonia* and *Rhodococcus*.

Chapter 9: Concluding remarks and future work.

Chapter 3 Materials and Methods

3.1. Sediment origin

Sediment core SG06 was extracted from the depocentre (35°35'08" N, 135°52'57" E) of Lake Suigetsu in 2006 by Nakagawa *et al*. (2012). The SG06 coring point was about 250 m offset to the north. Sediment coring was conducted using a hydro-pressure thin-walled piston sampler installed on a floating platform. Sediment core sections were extracted from the sampling tubes by using a mechanical piston. The entire sediment core was 74.2 m long and the inner diameter of the sampling tube was 7.8 cm. The extracted cores were immediately split into two half cylinders and a 'quasi-real scale' digital photograph of the freshly exposed core section surface was taken under natural daylight, before any colour changes through oxidisation could occur (observable within a minute of exposure to the air). A colour chart was placed alongside the sediment in each photograph and surface colour was instrumentally measured using a Konica Minolta CM-2002 Colourimeter at every 10 cm from the top of each core segment. The layers characteristics such as tephra layers, clay layers and coloured laminae were clearly identified at c. 10-20 cm intervals with assigned numbers. Sediment cores were obtained from 4 separate boreholes (A, B, C and D), all within 20 m horizontal distance from each other, with the core sections fully overlapping such that material from any given Lake Suigetsu sedimentary horizon would be represented by at least one of the individual sediment cores. Cores were wrapped with moist flower foam (Oasis^{TM}) (avoiding direct contact with the core material) using plastic (Saran) film to avoid desiccation. The wrapped cores were transported to cold storage (+4°C) near the lake as soon as possible. Cores were shipped using a cold container (+4°C) to the permanent cold storage site (+4°C) at the University of Newcastle, UK.

3.2. Sampling strategy 1- Studies of the distribution of bacterial communities throughout the lake sediment:

Sediment samples were taken every 4 m from upper core at 1.49 m down to the deepest core at depth 72.3 m. Table 3.1 illustrates the sampling strategy for this study, where the composite depth of pollen was set as the reference for sampling point in order to match to the pollen data. This was double checked using LevelFinder 4.6.1 software

created by Nakagawa (http://dendro.naruto-u.ac.jp/~nakagawa/) that enables the comparison of all the boreholes from A to D at once in terms of the depth, age, event layers, and various proxies (i.e. pollen data). In Table 3.1, the pollen site of B (N) 05 and A (S) 13 was excluded as the cores were heavily sampled, badly cracked or contaminated with fungus. Level Finder 4.6.1 software was used to determine and confirm the position of sampling points with matching pollen site and once identified, a double- L channel technique (Nakagawa, 2007) was employed to sample the core. This simple and non-destructive technique required only two L-channels and a fishing line. All the tools for sampling were sterilised prior to use. First, two sterile 1 cm × 1 cm × 10 cm L-channels were inserted one by one into the core surface at the targeted point, to form a 'u' shape (Figure 3a). Sterilised fishing line was slowly drawn through the sediment to give a clean cut off from the core. L-channels were then labelled with core number and depth before being removed from the core (Figure 3b). Sediment samples removed (Figure 3.1c) were wrapped with Saran cling film and sealed with black tubing to minimise contamination and subsequently stored at -80 ºC prior to fossil DNA extraction. This was repeated until all desired sampling points at every 4 m down the cores were sampled (Table 3.1).

Table 3.1: Sampling strategy 1 of which the 18 samples were taken at every 4m down the core depth.

Core No.	L-channels [Distance from core top (cm)]	Average distance from core top (cm)	Composite Depth (cm)	Age (BP)	Composite Depth of Pollen (cm)
A (N) 01	123.51- 133.58	128.545	148.54	567	129.03
A (N) 03	167.39- 177.52	172.455	599.52	4124	600.05
B (N) 05	25.12- 34.77	29.945	932.44	6860	-
B (N) 07	66.7- 77.02	71.860	1363.76	10911	1359.53, 1360.50, 1361.62, 1362.63, 1363.62, 1364.63, 1365.62, 1366.63, 1367.62, 1368.73
B (N) 11	16.3- 26	21.150	2150.45	22502	2150.04
A (S) 13	126.25- 136.55	131.400	2510.00	28911	-
A (N) 16	12.05- 22.07	17.060	2930.22	34945	2928.45
B (N) 17	87.21- 97.37	92.290	3359.29	41635	3355.75, 3356.73, 3362.791
B (N) 19	111.8- 119.9	115.850	3761.25	48376	3762.38
B (N) 21	115.27- 127.56	121.415	4197.09	60354	4198.37
A (N) 24	60.54- 70.57	65.555	4549.76	73990	4550.01
A (N) 28	45.8- 56	50.900	4998.20	88842	5000.01

Core No.	L-channels [Distance from core top (cm)]	Average distance from core top (cm)	Composite Depth (cm)	Age (BP)	Composite Depth of Pollen (cm)
A (S) 30	105.89- 115.87	110.880	5400.68	101916	5403.04
A (S) 32	112.5-122.3	117.400	5799.90	112998	5800.02
A (S) 35	51.11- 61.04	56.075	6199.88	121274	6200.08
A (N) 39	22.4- 32.48	27.440	6549.74	128993	6550.02
A (N) 43	45.3- 55.8	50.550	6946.65	136166	6950.07
A (N) 46	63.69- 73.88	68.785	7247.68	143019	7250.06

Core no. with A (N) 01 is the label given; A represents the borehole, N represents the direction of the core- where N (North) indicates the first 100 cm of the core and S (South) indicates the bottom 100 cm of the core. The number after the N or S represents the core number. Composite depth is calculated from the top core and composite depth of pollen is included to match with the core samples.

Figure 3.1: a) L-channels inserted into the point of sampling, b) labelling of core number and depth, c) L-channels removed from the core.

3.3. Sampling strategy 2- Studies of salinity effects on bacterial communities:

Sediment records of core SG06-A01 contained information of a seawater incursion event that occurred in 1664 AD (Kato *et al*., 2004). Sampling points were determined and confirmed using LevelFinder 4.6.1 software (http://dendro.naruto-u.ac.jp/~nakagawa/) before sediment samples were taken. Subsequently, sterile double L-channel technique (Nakagawa, 2007) was applied to produce a 1 m long core (1 cm × 1cm × 100 cm). The 1 m core was further sliced into 1 cm^3 using a centimeter slicer (code 1 to 20). All the sliced samples were kept in 15 ml sterile universal tubes and labelled accordingly. Samples were subsequently stored at -80ºC prior to fossil DNA extraction (Table 3.2).

Table 3.2: Sampling strategy 2 of which 20 samples were collected for the analysis of community in relation to salinity shift

SG06 Borehole	Sample code	Condition	Composite depth (cm)	Age (BP)
A01	1	3	77.60	335
A01	2	3	78.61	338
A01	3	3	79.62	341
A01	4	3	80.63	344
A01	5	3	81.64	348
A01	6	1	82.66	351
A01	7	1	83.67	354
A01	8	1	84.68	357
A01	9	1	85.69	361
A01	10	1	86.70	364
A01	11	1	91.40	379
A01	12	1	92.44	382
A01	13	1	93.47	386
A01	14	1	94.50	389
A01	15	1	95.54	392
A01	16	1	96.58	396
A01	17	1	97.61	399
A01	18	1	98.64	402
A01	19	1	99.68	405
A01	20	1	100.72	409

Condition 3 = sediment samples taken from the saline region.
Condition 1 = sediment samples taken from the freshwater region.

3.4. Sampling strategy 3- Studies of climatic effects on bacterial communities:

The event of climate transition from a cooler to warmer episodes was recorded in sediment core SG06-A09 (Nakagawa *et al*., 2005). LevelFinder 4.6.1 software (http://dendro.naruto-u.ac.jp/~nakagawa/) was utilised to confirm the position of targeted sediment depths and double- L channel technique (Nakagawa, 2007) was performed on the targeted area, with two 1 m L-channels sprayed with 70% ethanol. Sediment samples were further sliced into 1 cm^3 and subsequently stored at -80ºC prior to fossil DNA extraction (Table 3.3).

Table 3.3: Sampling strategy 3 of which 46 samples were collected for the analysis of community in relation to climatic change

SG06 Borehole	Sample Code	Condition	Composite depth (cm)	Age (BP)
A09	56	3	1696.10	14400
A09	57	3	1697.12	14411
A09	58	3	1698.15	14421
A09	59	3	1699.17	14434
A09	60	3	1700.19	14445
A09	61	3	1701.22	14458
A09	62	3	1702.24	14469
A09	63	3	1703.26	14481
A09	64	2	1704.28	17793
A09	65	2	1705.31	14504
A09	66	2	1706.33	14516
A09	67	2	1707.35	14531
A09	68	2	1708.38	14544
A09	69	2	1709.40	14558
A09	70	2	1710.41	14572
A09	71	2	1711.43	14588
A09	72	2	1712.44	14604
A09	73	2	1713.46	14618
A09	74	2	1714.47	14630
A09	75	2	1715.49	14642
A09	76	2	1716.50	14653
A09	77	2	1717.47	14664
A09	78	2	1718.43	14679
A09	79	2	1719.40	14695
A09	80	2	1720.50	14707
A09	81	2	1721.40	14719
A09	82	2	1722.30	14728
A09	83	2	1723.20	14740
A09	84	2	1724.10	14751
A09	85	2	1725.00	14762
A09	87	2	1726.80	14786
A09	88	2	1727.70	14799
A09	89	2	1728.60	14809
A09	90	2	1729.63	14824
A09	91	2	1730.67	14837
A09	92	2	1731.70	14852
A09	93	2	1732.70	14865
A09	94	2	1733.70	14881
A09	95	2	1734.70	14895
A09	96	1	1735.70	14910
A09	97	1	1736.70	14925
A09	98	1	1737.70	14939
A09	99	1	1738.70	14952
A09	100	1	1739.70	14967
A09	101	1	1740.70	14983

Condition 3 = sediment samples taken from the warmer climate.
Condition 2 = sediment samples in the transition zone
Condition 1 = sediment samples taken from the colder climate.

3.5. Sampling strategy 4- Comparison of parallel sediment cores:

Sampling points were determined and checked using LevelFinder 4.6.1 software (http://dendro.naruto-u.ac.jp/~nakagawa/). Two positions in core SG06-A03 at 146.11 cm and 154.32 cm were targeted alongside the identical points at other parallel cores of SG06-C05 (72.25 cm; 80.75 cm) and SG06-D03 (39.44 cm; 48.48 cm) (Table 3.4) with a sterile syringe as an extraction tool. Sediment samples were labelled and stored in a sterile 15 ml universal tube. Samples were subsequently stored at -80°C prior to fossil DNA extraction.

Table 3.4: Horizontal sampling at the same point taken from 3 different boreholes- A, C and D

SG06 Borehole	Sample code	Depth from core top (cm)	Average of Composite Depth (cm)
A03	1	145.26	573.02
C05	1	72.25	573.30
D03	1	39.44	573.19
A03	2	154.32	582.14
C05	2	80.75	582.20
D03	2	48.48	582.23

Core A03-1, C05-1, D03-1 have the same depth while core A03-2, C05-2 and D03-2 are another similar point taken at the same depth. Borehole B was not taken because most of the regions were disturbed.

3.6. Chemical data of the sediments

Sediment samples were weighed out at 0.30 g in sterile microcentrifuge tubes and air-dried in a drying cabinet for a day with the lids slightly opened. Subsequently, the dried sediment samples were ground with mortar and pestle, and passed through a 0.5 mm sieve before transferring to a new sterile microcentrifuge tube. The prepared sediment samples were labelled and sent to Newcastle University for various chemical analyses, including total nitrogen (TN), total organic carbon (TOC), and sulphur (S). Other chemical data, such as $\delta^{15}N$, density, water content, biogenic silica oxide ($BioSiO_2$) and iron (III) oxide (Fe_2O_3) were obtained from the specific site created for Lake Suigetsu project at http://kairos.naruto-u.ac.jp/~suigetsu/ with authorised access only.

3.7. Fossil DNA extraction:

The fossil DNA from the collected sediment samples of SG06-A01-1 to A01-55 and A09-56 to A09-101 were extracted using PowerLyzer PowerSoil DNA Kit (MoBio, California, USA) according to the manufacturer's instructions.

3.8. Polymerase chain reaction (PCR):

3.8.1. Amplification of bacterial 16S rRNA genes

To discover the total bacterial community in the sediment, 2.5 µl of 10 µM primers V3f and 10 µM V3r were used for amplification (Muyzer *et al*., 1993; Table 3.5). A high fidelity polymerase (*Pfx*), possessing a proofreading capability from 3'-5' was utilised to minimise the formation of chimera (Lahr and Katz, 2009). The 50 µl PCR master mix included 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM deoxynucleoside triphosphate (dNTPs, made up of 5 µl of 100 mM dATP, dCTP, dGTP and dTTP respectively), 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of DNA template (extracted DNA) and 32.4 µl of sterile 18.2 Ω H_2O. DNA was denatured at 97˚C for 5 minutes prior to amplification with 19 cycles of denaturation at 96˚C for 1 minute, annealing temperature at 65˚C for 1 minute with a -0.5 touchdown temperature, elongation at 68˚C for 30 seconds and 14 cycles of denaturation at 94˚C for 1 minute, annealing for 1 minute at 55˚C and elongation at 68˚C for 3 minutes. Final extension at 68˚C was run for 10 minutes. Products were further amplified with a 40 bp GC-clamp V3fC primers (Muyzer *et al*., 1993; Table 3.5). A negative control was set up for contamination check for each PCR cycle.

3.8.2. Amplification of Alphaproteobacteria 16S rRNA gene

To detect *Alphaproteobacteria* community, primers F203a (Gomes *et al*., 2001; Table 3.5) and R-Act (Heuer *et al*., 1997; Table 3.5) were used. The PCR master mix consisted of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 1.0 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of DNA template (extracted DNA) and 31.9µl of sterile 18.2 Ω H_2O. The amplification cycles have a total of 35 PCR cycles, with an initial denaturation at 94°C for 5 minutes, followed by 1 minute of

denaturation at 94°C, 1 minute of primer annealing at 56°C, 2 minutes of elongation at 68°C and a final extension at 68˚C for 10 minutes. PCR products were then clamped using V3fC and V3r primers (Table 3.5) and the cycling process was run at the same conditions as the *Eubacteria* PCR 3.7.1. A negative control was set up for contamination check for each PCR cycle.

Table 3.5: Bacterial and archaeal primers sets

Target group	**Primer name**	**Primer sequence (5'- 3')**	**Reference**
Bacteria	V3f	CCT ACG GGA GGC AGC AG	Muyzer *et al*. (1993)
	V3fC	CGC CCG CCGC CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC	Muyzer *et al*. (1993)
	V3r	ATT ACC GCG GCT GCT GG	Muyzer *et al*. (1993)
Alphaproteobacteria	F203a	CCG CAT ACG CCC TAC GGG GGA AAG ATT TAT	Gomes *et al*. (2001)
	R-Act	TAC GGY TAC CTT GTT ACG ACT T	Heuer *et al*. (1997)
Betaproteobacteria	Beta359f	GGG GAA TTT TGG ACA ATG GG	Mühling *et al*.
	Beta682r	ACG CAT TTC ACT GCT ACA CG	Mühling *et al*.
	518f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CAG CAG CCG CGG TAA T	Muyzer *et al*. (1993)
Deltaproteobacteria	DSR2060f	CAA CAT CGT YCA YAC CCA GGG	Geets *et al*. (2006)
	DSR1f	ACS CAC TGG AAG CAC G	Geets *et al*. (2006)
	DSR2060f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CAA CAT CGT YCA YAC CCA GGG	Geets *et al*. (2006)
	DSR4r	GTG TAG CAG TTA CCG CA	Geets *et al*. (2006)
Gammaproteobacteria	Gamma395f	CMA TGC CGC GTG TGT GAA	Mühling *et al*.
	Gamma871r	ACT CCC CAG GCG GTC DAC TTA	Mühling *et al*.
	581f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCA GCA GCC GCG GTA AT	Muyzer *et al*. (1993)
	785r	CTA CCA GGG TAT CTA ATC C	Lee *et al*. (1993)
Acidobacteria	Acid31F	GAT CCT GGC TCA GAA TC	Barns *et al.* (1999)
	907R	CCG TCA ATT CMT TTG AGT TT	Muyzer *et al*. (1998)
Actinobacteria	SC-Act-235a	CGC GGC CTA TCA GCT TGT TG	Stach *et al*. (2003)
	SC-Act-878a	CCG TAC TCC CCA GGC GGG G	Stach *et al*. (2003)
Bacteroidetes	CFB555f	CCG GAW TYA TTG GGT TTA AAG GG	Mühling *et al*.
	CFB968r	GGT AAG GTT CCT CGC GTA	Mühling *et al*.
	CFB555f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCG GAW TYA TTG GGT TTA AAG GG	Mühling *et al*. (2008)
	907R	CCG TCA ATT CMT TTG AGT TT	Muyzer *et al*. (1998)
Cyanobacteria	CYA361f	GGA ATT TTC CGC AAT GGG	Mühling *et al*.
	518f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCA GCA GCC GCG GTA AT	Muyzer *et al*. (1993)
	CYA785r	GAC TAC WGG GGT ATC TAA TCC	Mühling *et al*.
Firmicutes	Firm350f	GGC AGC AGT RGG GAA TCT TC	Mühling *et al*.
	Firm814r	ACA CYT AGY ACT CAT CGT TT	Mühling *et al*.
	518f-GC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCA GCA GCC GCG GTA AT	Muyzer *et al*. (1993)
	785r	CTA CCA GGG TAT CTA ATC C	Lee *et al*. (1993)
Archaea	Arc344f	ACG GGG CGC AGC AGG CGC GA	Bano *et al*. (2004)
	Arc344f-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG	Bano *et al*. (2004)
	517R	ATT ACC GCG GCT GCT GG	Bano *et al*. (2004)

Target group	Primer name	Primer sequence (5'- 3')	Reference
16S	27f	AGA GTT TGA TCC TGG CTC AG	Lane (1991)
	1525R	AAG GAG GTG ATC CAG CC	Lane (1991)

3.8.3. Amplification of Betaproteobacteria 16S rRNA gene

For the detection of the *Betaproteobacteria* community, primers including Beta359f and Beta682r were used (Mühling *et al*., 2008; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 32.4 µl of sterile 18.2 Ω H_2O. The amplification cycles involved an initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 96°C for 1 minute, 63°C of annealing temperature for 1 minute and elongation at 74°C for 1 minute. The cycle was ended with a final extension at 74˚C for 10 minutes. PCR products were then clamped using primers 518f-GC (Muyzer *et al*., 1993) and Beta682r (Table 3.5). This semi-nested cycling condition was similar to the first cycle, with the exception of the annealing temperature of 60°C. A negative control was set up for contamination check for both PCR cycles.

3.8.4. Amplification of Deltaproteobacteria 16S rRNA gene

For the detection of the *Deltaproteobacteria* community, primers including DSR2060f and DSR4r were used (Geets *et al*., 2006; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 32.4 µl of sterile 18.2 Ω H_2O. The amplification cycles included with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing temperature at 55°C for 1 minute and elongation at 72°C for 1 minute. The cycle was ended with a final extension at 72˚C for 10 minutes. PCR products were then clamped using primers DSR2060f-GC and DSR4r (Geets *et al*., 2006; Table 3.5), with the same semi-nested cycling conditions. A negative control was set up for contamination check for both PCR cycles.

3.8.5. Amplification of *Gammaproteobacteria* 16S rRNA gene

Gamma 395f and Gamma 871r primers were used to detect the presence of the community of *Gammaproteobacteria* (Mühling *et al*., 2008; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 1.0 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 31.9 µl of sterile 18.2 Ω H_2O. The amplification cycles started off with an initial denaturation at 96°C for 4 minutes, followed by a 30-cycle of denaturation at 96°C for 1 minute, annealing temperature at 54°C for 1 minute and elongation at 74°C for 1 minute. The cycle was completed with a final extension at 74˚C for 10 minutes. PCR products were subsequently clamped with 518f-GC and 785r (Lee *et al*., 1993) (Table 3.5), with a semi-nested cycling condition similar to the first cycle and a slightly different annealing temperature of 56°C. A negative control was set up for contamination check for both PCR cycles.

3.8.6. Amplification of *Acidobacteria* 16S rRNA gene

To detect the community of *Acidobacteria*, primers Acid31F (Barns *et al.*, 1999; Table 3.5) and 907R (Muyzer *et al*., 1998; Table 3.5) were exploited. The PCR master mix consisted of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 1.0 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of DNA template (extracted DNA) and 31.9 µl of sterile 18.2 Ω H_2O. The following thermocycler conditions were used, a total of 35 PCR cycles, with an initial denaturation at 94°C for 5 minutes, followed by 1 minute of denaturation at 94°C, 1 minute of primer annealing at 50°C and 2 minutes of elongation at 68°C. The cycle was ended with a final extension at 68˚C for 10 minutes. PCR products were then clamped using V3fC and V3r primers (Table 3.5) and the cycling process was run at the same conditions as stated in the section 3.7.1. A negative control was set up for contamination check for both PCR cycles.

3.8.7. Amplification of Actinobacteria 16S rRNA gene

The *Actinobacteria* community was detected using the primers sets SC-Act-235a and SC-Act-878a (Stach *et al*., 2003; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of

each of the 10 µM F' and R' primers, 1.0 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 31.9 µl of sterile 18.2 Ω H_2O. The cycling conditions comprised of an initial denaturation at 95°C for 4 minutes, followed by denaturation at 95°C for 45 seconds, annealing at 72°C for 45 seconds and extension at 72°C for 1 minute. A 'touchdown' protocol was included from the preceding cycle with each cycle decreased by 0.5°C; subsequently followed by 15 cycles of 95°C for 45 seconds, 68°C for 45 seconds and 72°C for 1 minute, with the last cycle followed by a 5 minutes extension at 72°C. PCR products were then clamped using V3fC and V3r primers (Table 3.5) and the cycling conditions were the same as the bacteria method as described on 3.7.1. A negative control was set up for contamination check for both PCR cycles.

3.8.8. Amplification of Cyanobacteria 16S rRNA gene

For the detection of the community of *Cyanobacteria*, primers including CYA361f and CYA785r were employed (Mühling *et al*., 2008; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 32.4 µl of sterile 18.2 Ω H_2O. The cycling conditions involved an initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 96°C for 1 minute, 59°C of annealing temperature for 1 minute and elongation at 74°C for 1 minute. The cycle was ended with a final extension at 74˚C for 10 minutes. PCR products were then clamped with primers sets of 518f-GC (Muyzer *et al*., 1993) and CYA785r (Table 3.5). This semi-nested cycling conditions used were similar to the first round of amplification, except for the annealing temperature which was at 56°C. A negative control was set up for contamination check for both PCR cycles.

3.8.9. Amplification of *Bacteroidetes* 16S rRNA gene

For the detection of the *Bacteroidetes* community, primers including CFB555f and CFB968r were used (Mühling *et al*., 2008; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3U/µl *Pfx* polymerase, 1 µl of extracted DNA as the

template and 32.4 µl of sterile 18.2 Ω H_2O. The cycling conditions comprised of an initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 96°C for 1 minute, 61°C of annealing temperature for 1 minute and elongation at 74°C for 1 minute, with a final extension step at 74˚C for 10 minutes. PCR products were then clamped with CFB555f-GC (Mühling *et al*., 2008) and 907r (Table 3.5).This semi-nested cycling condition was similar to the first cycle, with the exception of the annealing temperature of 64°C. A negative control was set up for contamination check for both PCR cycles.

3.8.10. Amplification of *Firmicutes* 16S rRNA gene

For the detection of *Firmicutes* community, primers used were Firm350f and Firm814r (Mühling *et al*., 2008; Table 3.5). The PCR master mix comprised of 5 µl of 10 × PCR amplification buffer, 5 µl of 10 × PCR enhancement solution, 2.5 µl of each of the 10 µM F' and R' primers, 0.5 µl of 50 mM $MgSO_4$, 0.6 µl of 25 mM dNTPs, 0.5 µl of 3 U/µl *Pfx* polymerase, 1 µl of extracted DNA as the template and 32.4 µl of sterile 18.2 Ω H_2O. The thermocycler conditions comprised of an initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 96°C for 1 minute, 57°C of annealing temperature for 1 minute and elongation at 74°C for 1 minute, with a final extension of 74˚C for 10 minutes. PCR products were then clamped with 518f-GC and 785r primers (Table 3.5). This semi-nested cycling condition was similar to the first cycle, with the exception of the annealing temperature of 56°C. A negative control was set up for contamination check for both PCR cycles.

3.8.11. Amplification of archaeal 16S rRNA gene

The Archaea primers set, Arc344f, Arc344f-GC and 517R (Bano *et al*., 2004; Table 3.5) were used for the amplification of the *Archaea* community. The amplification steps were similar to the previous bacterial analyses. The master mix was as described on 2.7.1 with additional 2.5 µl of 10 mg/ml bovine serum albumin (BSA) and MgSO4 concentration at 0.6 mM. The cycling conditions used were an initial denaturation at 94˚C for 5 minutes, followed by 10 cycles of 94˚C denaturation for 30 seconds, annealing at 61˚C for 30 seconds with a -0.5 touchdown temperature, elongation at 68˚C for 1 minute and 25 cycles of 94˚C denaturation for 30 seconds, annealing at 56˚C for 30 seconds and elongation at 68˚C for 1 minute. The amplification was ended with a final extension at 68˚C for 10 minutes. A negative

control was set up for all PCR reactions to ensure that the reaction mix and components were not contaminated.

3.9. Confirmation of successful PCR amplification:

The confirmation of successful PCR amplified products was achieved using 1% (w/v) agarose gel electrophoresis (AGE) in 1 × TAE (Tris-acetate EDTA) buffer (Appendix 1a) with 5 µl of SYBR safe (Invitrogen; 10,000 × in DMSO). The 1% (w/v) agarose gel was submerged in 1 × TAE buffer and an aliquot of 6 µl of 1 kb Hyperladder I (Bioline; Appendix 2) was loaded onto the first well as the marker, followed by 5 µl aliquot of PCR products mixed with 1 µl of 6 × Bromophenol Blue (BPB) (Appendix 1a) which were loaded into the rest of the wells. The agarose gel was electrophoresed at 120 mA for 22 minutes. The agarose gel was removed and viewed under UV transilluminator on BIO-RAD gel doc 2000 using Quantity One^{TM} 4.1.1 software. Finally, the fragment size of the PCR products were compared to the fragments from Hyperladder I.

3.10. Analysis of bacterial community using denaturing gradient gel electrophoresis (DGGE):

3.10.1. Preparation of DGGE gels

Two glass plates were cleaned using 50% (v/v) ethanol to remove any residue before casting DGGE gels. A thin film of silicon grease was applied to the inner edges of the spacers that separate the glass plates to prevent leakage of the denaturant during the process of gel casting (Brinkhoff and van Hannen, 2001). The large glass plate (20 cm × 16 cm) was placed before the smaller glass plate (16 cm × 16 cm) on the bench to ensure they were properly aligned by using alignment card before glass plates were secured with plastic brackets. Two gels were usually set up to be electrophoresed on the same run and if only one gel was needed, an empty balance plate (without gel) was prepared without spacers in between plates to prevent the leaking of buffer from the upper chamber during electrophoresis.

3.10.2. Casting DGGE gels

After assembling the plates, a 19 gauge needle fitted with a tube and Y-fitting was attached to the centre and in between the plates. Two different gradients of denaturants, as summarised in the table below were prepared and mixed in a sterile 50 ml universal tube.

Table 3.6: Reagents for casting DGGE gels

Reagent	High (55%)	Low (35%)
Dcode dye (Appendix 1b)	100 µl	0
Denaturing solution (Appendix 1b)	25 ml	25 ml
APS (10% w/v)	216 µl	216 µl
TEMED	21.6 µl	21.6 µl

Two syringes with rubber tubing were used to draw 16 ml of the solution each from the tubes. The syringes were secured in a Model 475 gradient former and the solutions were pumped into the gel cast by turning the cam wheel in a constant pace to ensure for an equal gradient. After the solutions had been dispensed, the needle was removed and a 16-well comb was inserted into the gel cast and the solution was allowed to set for two hours at room temperature.

3.10.3. Gel electrophoresis

The buffer tank was first filled with 7 L of 1 × TAE (Appendix 1a). The DGGE control unit was placed in the buffer tank and the buffer was set to heat up at 60ºC with constant stirring. Once the buffer was at 60ºC, the control unit was removed and the gel casts were lowered into the buffer chamber. The upper chamber and the buffer tank were filled with 1 × TAE buffer to the marked line. A 15 µl aliquot of sample (PCR products) was mixed with an equal volume of 2 × DGGE loading buffer (Appendix 1b) and loaded into the well. Subsequently, the DGGE control unit was replaced and the temperature was allowed to return to 60ºC. The DGGE gels with samples loaded were run at 200 V for 4.5 hours.

3.10.4. Gel staining and viewing

The gels removed from the glass plates were stained with an aliquot of 25 µl of SYBR Green I (Invitrogen; 10,000 ×), in 1 × TAE buffer (Appendix 1a) in a staining tank, whilst being agitated for 30 minutes. The gels were later de-stained by washing in distilled water to remove any excess stain and subsequently viewed under UV

transilluminator (Bio-Rad Gel Doc 2000) and photographed using the Quantity One™ 4.1.1 software (Bio-Rad).

3.11. Statistical analysis of DGGE gels:

3.11.1. Analysis of DGGE profiles

DGGE gels image of each profile were analysed using Phoretix 1D Pro and TotalLab software to manually assign and match each of the bands on DGGE gels. Phoretix 1D Pro allowed multiple gels of the same set of data to be analysed and matched at the same time. Lane background was subtracted by using a rolling disc algorithm and the lane were normalised so that all lanes contained the same amount of total signal. Lane profiles were corrected for differences in migration rate by manually assigning R_f lines to marker lane bands (Tourlomousis *et al*., 2010). The output of matching DGGE data across the gels were presented in the form of dendrogram or tables to allow the comparison of bands and lanes similarities. The band matrix was input into a Microsoft Excel spreadsheet and the total intensity for each lane was calculated by dividing the normalised band values per lane by the total relative band intensity which was used for the calculations of different statistical analyses.

3.11.2. Analysis of microbial communities

To investigate the structure of the microbial communities present in the sediment records of Lake Suigetsu, species richness, evenness and Shannon diversity were calculated using the normalised DGGE band matrix. Species richness (Rr) was calculated by counting the total number of bands present in each sample from the DGGE band matrix. The Shannon diversity index (H') was calculated using the function in Microsoft Excel following the formula below;

$$H' = -\Sigma\ (pi \log[pi])$$

The symbol *pi* = the relative intensity of each band in a lane. The sum of the outcome values (-Σ) for each lane, multiplied by -1, is the Shannon diversity of the sample. Species evenness (E) was further calculated using Microsoft Excel by dividing *H'* by the log of R.

3.11.3. Principle component analysis (PCA)

PCA as the basic for multivariate data analysis was carried out for each taxa community profile using CANOCO for Windows 4.5. The data were standardised to eliminate the effect of the different size and the unit of measurement. PCA was used to reduce the data set to only two major components, PC1 and PC2. The first principle component (PC1) axis usually accounts for the largest eigenvalue and explains the greatest variance in the data set while the second component (PC2) axis accounts for the remaining amount which will be the second greatest (Hammer *et al*., 2001).

3.11.4. Ecological analysis

The normalised band matrix of DGGE microbial profiles were analysed using CANOCO for Windows v. 4.5.1. Species data set and environmental data set were first converted to a compatible format for CANOCO using WCanoImp (4.5.2.0). The normalised band matrices were first analysed by detrended correspondence analysis (DCA) to check the length of the gradient. If the gradient axis length was less than 3.5, a direct gradient method (redundancy analysis, RDA) will be employed for the set of microbial data. A unimodal method of canonical correspondence analysis (CCA) in combination of Monte Carlo permutation test will be utilised when the gradient axis length is larger than 3.5. The result from the Monte Carlo permutations was statistically significant if the p-value was < 0.05. The analysed data were plotted in CANODRAW and a biplot was created for samples and environmental variables. The discrete variables were denoted by a centroid while continuous variables were represented by an arrow.

3.11.5. Pearson correlation coefficient

Pearson Correlation Coefficient was determined using MiniTab to evaluate the relationship between microbial Shannon diversity and environmental parameters. The data was analysed under ‘Basic Stats; Correlation’. A positive coefficient value indicated a positive correlation between microbial diversity and environmental variables while a negative coefficient value represented a negative relationship. A significant effect was indicated by p-value < 0.05.

3.11.6. Raup-Crick similarity index

The Raup-Crick (S_{RC}) similarity index was calculated using the Monte Carlo procedure to measure the presence/absence of band between two samples by calculating the distribution of co-occurrences from 200 random replicates using PAST programme (Hammer *et al*. 2001). A value of 1 refers to two identical samples while a value of 0 refers to two completely unrelated samples. The output also generates an indicator of statistical significance where a value of ≤ 0.05 indicates that the two samples are less similar than would be expected by chance and a *p*-value of ≥ 0.95 indicates that the samples are more closely related then would be expected by chance.

3.11.7. 454 pyrosequencing for metagenomics study

The extracted fossil DNA from sediment samples for salinity and climatic shift studies were selected for a metagenomics study. Sediment samples SG06 A01- 1, 2, 3, 4, 5, 6, 7, 12a, 12b, 19 and 20 were selected for the study of salinity shift while sediment samples SG06 A09- 57, 58, 59, 67, 76, 84, 93, 99, 100 and 101 were chosen for the climatic shift study. The chosen sediment samples were sent to Research and Testing Laboratories (RTL) LLC. (Lubbock, Texas, U.S.) for 454 pyrosequencing. Assay b.5: 341F-907R was selected to target the V3-V5 hypervariable regions of the 16S rRNA gene given that these regions have been reported to generate better DGGE profiles than the other hypervariable regions (Yu and Morrison, 2004). Primer sets of 341F (5′-CC TAC GGG AGG CAG CAG-3′) and 907R (5′-CC GTC AAT TCC TTT GAG TTT-3′) were used and the DNA pyrosequencing was performed using the 454 Genome Sequencer FLX Titanium platform (Roche) based on previously described methods (Dowd *et al*., 2008).

3.11.8. Statistical analysis for metagenomics study

The sequencing reads of the metagenomics data were checked and quality filtered using the split-library.py script in QIIME 1.6.0 (Caporaso *et al*., 2010). First, the sequencing reads were screened if they matched to the bar coded tags with no ambiguous bases and subsequently checked for mismatches to primer sequences. The read-lengths of the sequences were also screened if they fell between 200 – 700 base pairs and possessed an average quality score of > 25 in a sliding window of 50 bp. The quality sequences were then clustered into operational taxonomic units (OTUs) using

UCLUST (Edgar, 2010) at 97% similarity. Representative sequences for each OTU were aligned using PyNAST (Caporaso and Bittinger, 2010) and taxonomic identities were assigned using the RDP-classifier (version 2.2) (Wang *et al*., 2007) with 50% as confidence threshold value before chimeric sequences were checked using ChimeraSlayer (Haas *et al*., 2011). Finally, the metagenomics data were analysed using multivariate partial least squares discriminant analysis (PLS-DA) using SIMCA 13.0 (Umetrics, Stockholm, Sweden) (Eriksson *et al*. 2006). Hotelling's T^2 *t*-test at 0.95 was set to check if the data fell within the confidence limits.

3.12. Isolation and characterisation of bacteria:

3.12.1. Extraction of bacteria from sediment samples using dispersion and differential centrifugation (DDC)

The sediment samples were weighed out (0.25 g) in duplicate into sterile 15 ml centrifuge tube. Aliquots of 500 µl of 0.1 % w/v sodium cholate (Appendix 1c), 0.5 g of chelating resin (Fluka Analytical) and 5 sterile glass beads (2-3 mm diameter) were added to both tubes to aid in effective and efficient shaking. The two 15 ml centrifuge tubes were mixed for 2 hours using Stuart Rotator SB2. After 2 hours of shaking, the centrifuge tubes were centrifuged at 500 × g for 2 minutes, the supernatant was collected and labelled as Fraction A. To the pellets, 500 µl of 0.05 M cold Tris-buffer (pH 7.4) (Appendix 1c) was added and mixed using rotator for 1 hour. After centrifugation at 500 × g for 1 minute, the supernatants were collected and pooled with the previous supernatants labelled Fraction A.

Next, 500 µl of 0.1 % w/v sodium cholate was added to each pellet and mildly sonicated using DAWE Sonicleaner at 240 V for 1 minute. An aliquot of 500 µl of 0.1 % w/v sodium cholate was added to each suspension and gently mixed for 1 hour. The supernatants were collected after centrifugation at 500 × g for 1 minute and dispensed into a new sterile 15 ml centrifuge tube labelled as Fraction B. Both pellets were later resuspended with 500 µl of 0.05 M cold Tris-buffer (pH 7.4) and mixed for 1 hour. The second supernatant aliquots of Fraction B were collected after centrifugation at 500 × g for 1 minute and added to Fraction B. Subsequently, the pellets were resuspended with 1.5 ml of sterile cold distilled water and agitated for 1 hour followed by centrifugation at 500 × g for 1 minute to collect the supernatants, labelled as Fraction C. Aliquots of

1.5 ml of distilled water was added to both pellets, resuspended and mixed for a further hour. Both suspensions were centrifuged at 500 × g for 1 minute and the supernatants were pooled and added to Fraction C. Lastly, the pellets were resuspended with 1 ml of sterile ¼ strength of Ringer's solution (Appendix 1c) and agitated for 30 minutes and the suspensions were allowed to settle for 10 minutes before being transferred by pipette to a 1.5 ml centrifuge tube labelled Fraction D and the remaining residues were labelled as Fraction E.

3.12.2. Isolation of lake sediment bacteria

Difco Marine Agar 2216 (MA; Appendix 3; Pathom-aree *et al*., 2006) and Trypticase Peptone Yeast Agar (TPA; modified from Smith *et al*., 1982) media were used for aerobic cultivation, while for anaerobic cultivation, nitrate mineral salts (NMS; Dworkin *et al*., 2006) medium and nitrate mineral salts containing artificial sea salts (ASS-NMS; Dworkin *et al*., 2006) medium were selected (see Appendix 3 for all media formulations). For aerobic cultivation, the collected fractions of B (N) 05, B (N) 07 and A01 were serially diluted in sterile ¼ strength of Ringer's solution (Appendix 1c) to 10^{-4}, aliquots 200 µl from dilutions were plated in triplicate onto each medium, MA and TPA, which consisted of anti-fungal antibiotics, nystatin (50 µg/ml) (Appendix 1d). For anaerobic cultivation, only fractions from B (N) 05 were used. Each fraction was serially diluted to 10^{-2} and plated in duplicate onto NMS and ASS-NMS with both media contained of 50 µg/ml of nystatin as final concentration. These anaerobic culture plates were sealed in candle jars with a gas pack inside. Both the aerobic and anaerobic plates were incubated at 25°C for a minimum of one week time. Anaerobic plates were incubated for a longer period of at least 4 weeks.

3.12.3. Characterisation of bacterial macro-morphology

The colonial pigmentation of isolates were observed by eye and recorded according to the National Bureau of Standards (NBS) Colour Name Charts (Kelly 1958; NBS 1964). Their form, elevation, margin and colony diameter were also examined and recorded (Appendix 4). Each morphological type was selected for purification and storage. They were then streaked out onto TPA and MA media without nystatin and cultured for 3 days at 25°C. Once purity was confirmed, the pure culture was stored in 20 % v/v glycerol suspensions (Appendix 1d) in duplicates with one stored at -20°C while the other at -80°C for long term preservation.

3.12.4. Characterisation of bacterial micro-morphology

3.12.4.1. Gram staining

Isolates were Gram-stained after 1 day of incubation at 25˚C. Isolates were first, heat-fixed onto microscope slides and then flooded with crystal violet (or Methylene blue) for 1 minute. The slide was gently rinsed with flowing tap water for 2 seconds. Iodine was then flooded onto slide and was allowed to remain for 1 minute before rinsing off with flowing tap water. The slide was decolourised with acetone for 30 seconds with the slide held slanting and stopped when the runoff became clear. Finally excess decolourizer was gently washed off with flowing tap water and slide was then counterstained with safranin for 30 seconds. Excess counterstain was removed by gentle flow of tap water and slide was blot-dried and left to dry. Subsequently, the slides were examined under light microscope from 4 × to 100 × under oil emersion. Gram-positive bacteria stain blue/purple while gram-negative bacteria will stain pink/red.

3.12.4.2. Acid-fast staining

Isolates that possessed a filamentous form were chosen for this test. First, the isolate was heat-fixed onto a slide, then a strip of blotting paper was placed over the slide to allow saturation of Ziehl's carbol fuchin solution (Sigma-Aldrich). The slide was allowed to sit on top of steam of boiling water for 3 to 5 minutes. Ziehl's carbol fuchin solution was re-applied once dried out. Blotting paper was removed after 5 minutes and the slide was rinsed with flowing water gently until runoff was clear. Subsequently, the slide was flooded with acid alcohol, decolourised for 10 to 15 seconds and then rinsed off. Lastly, the slide was counterstained with crystal violet for 1 minute. After the slide was rinsed, blotted and dried, it was examined under light microscope under 100 × magnifications.

3.13. Analysis of 16S rRNA genes:

3.13.1. Bacterial DNA extraction

The total genomic DNA of representative isolates from brackish sediment (SG06-A01) was extracted using a modified protocol from Sambrook *et al*. (1989). A loopful of biomass was suspended into 150 µl of 1 × TE buffer (pH 8) (Appendix 1e), 2.5 µl of lysozyme (50 µg/ml) and 10 µl proteinase K (10 µg/ml). The mixture was mixed by

vortex after every preparation and incubated at 37°C for 2 hours. The mixture was then centrifuged at maximum speed of 13,000 rpm for 5 minutes. The supernatant was carefully transferred to avoid transferring of pellet into a sterile microcentrifuge tube. The supernatant was then incubated at 75°C for 15 minutes and centrifuged for 5 minutes at maximum speed of 13,000 rpm. Approximately, 100 µl supernatant containing the genomic DNA was transferred into a sterile microcentrifuge tube and stored at -20°C prior use. This genomic DNA extraction method used for the 19 representative isolates selected for further phenotypic characterisation and description were different. The protocols were carried out using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) based on the manufacturer's instructions.

3.13.2. Amplification of bacterial 16S rRNA genes

The 16S rRNA genes of the representative isolates were amplified using 27f and 1525R primers (Lane, 1991; Table 3.5). The 50 µl of PCR reaction mixture comprised of 5 µl of 10 × Taq buffer, 1 µl of 10 mM dNTPs, 0.5 µl of 100 mM F' and R' primers, 0.25 µl of 5,000 U/ml Taq polymerase, 1 µl of extracted DNA and 41.75 µl of sterile 18.2 Ω H_2O. A negative control was set up as well to detect contamination. The cycling condition initiated with an initial denaturation at 95°C for 3 minutes, and 31 PCR cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and elongation at 68°C for 90 seconds, with a final extension at 68°C for 10 minutes. Aliquots of 5 µl of PCR products were mixed with 1 µl of 6 × BPB (Appendix 1a) and were run on AGE to check for contamination and to determine if PCR was successful.

3.13.3. Purification of amplified 16S rRNA genes

An aliquot of 5 µl of amplified 16S rRNA genes was mixed with 0.5 µl of Exonuclease I (10 u; Thermo Scientific) and 1 µl of FastAP Thermosensitive alkaline phosphatase (1 u; Thermo Scientific). The mixture was incubated at 37°C for 15 minutes and the reaction was terminated by heating the mixture to 85°C for 15 minutes.

3.13.4. Phylogenetic analysis

The results of 16S rRNA sequences obtained from GENEIUS (Newcastle University) were assembled using MicroSeqTM automated software (assessed in Newcastle University). The reassembled sequences were checked for possible identity

or the nearest match of each sequence via BLAST (Basic Local Alignment Search Tool) search at NCBI (National Centre for Biotechnology Information) website. The strains were further categorised into 3 major groups; the Gram-positive, Gram-negative and high G+C Actinobacteria in 3 different files generated using MEGA 5.0 software (Tamura *et al*., 2011). The 16S rRNA sequences (in FASTA format) of the strains from the same suborder (obtained from the LPSN site- http://www.bacterio.net/) were included in the analysis for comparison and the 16S rRNA gene sequence of *E.coli* was used as an anchor strain for uniform sequence alignment. All the 16S rRNA sequences were aligned using CLUSTAL W and the aligned sequences were utilised to generate phylogenetic trees and similarity matrices. The phylogenetic analyses carried out including nucleotide difference analysis based on pairwise deletion method, *p*-distance analysis, Jukes and Cantor analysis and finally the phylogenetic tree was constructed using Neighbour-Joining algorithm. For Chapter 8, the phylogenetic trees were constructed based on three tree-making algorithms namely, maximum-likelihood, maximum-parsimony and neighbour-joining algorithms.

3.14. Phenotypic characterisation:

3.14.1. Preparations of strains

Novel representative isolates from the phylum *Actinobacteria* were selected for biochemical characterisation. There were five strains from genus *Dermacoccus*, one belonging to the genus *Dietzia*, one falling within the evolutionary radition of the genus of *Leifsonia* and two belonging to the genus *Rhodococcus* (see Appendix 5 for isolates and its 16S rRNA gene sequences). All these test strains alongside 30 representative type strains (Appendix 6) were cultured on glucose yeast extract agar (GYEA) medium (Appendix 7a) for a minimum of 3 days at 28°C. A loopful of biomass from each strain was mixed with sterile distilled water in 7 ml sterile bijoux bottles to prepare the suspensions in 5.0 McFarland densities (Appendix 7b; McFarland, 1907). Aliquots of 1 µl of the bacterial suspension were spotted onto agar plates whilst 5 µl of the bacterial suspensions were used to inoculate the chemical tests prepared in bijou tubes. Each spotted plate held 10 different strains and spotting strategy was based on a drawn template (Appendix 8) to ensure even distribution. All the tests were carried out in duplicate. Strains were compared to control media plates and were scored positive if growth on the test plate was equal to or greater than that on the positive control plate, or

greater than on the negative control plates (+); the test was scored negative if growth was equal to or less than that on the negative control plates (-).

3.14.2. Colonies pigment and description

The test strains and the representative type strains were grown on GYEA medium (Appendix 7a) at 28°C for 10 days and examined by eye to detect colonial shape (form, margin and elevation), texture and pigmentation properties. National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1958; NBS, 1964) were used to record the colours of the colonies.

3.14.3. Biochemical analyses

3.14.3.1. Temperature Test

Bacterial suspension of each strain was spotted on GYEA medium (Appendix 7a) in duplicate. Culture plates were allowed to dry before incubation at different temperature (10°C, 28°C, 37°C and 45°C) for 14 days. Temperature ranges were determined from former studies. The culture plates were compared to the control plate and the presence of growth was scored positive.

3.14.3.2. pH Test

GYEA media with various pH were prepared at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10 in duplicate using buffered solutions (Appendix 7a). These wide ranges of pH conditions were determined from past studies. After inoculation, culture plates were allowed to dry and incubated at 28°C for 14 days. The culture plates were compared to the control plate (at pH 7) and the presence of growth was scored positive.

3.14.3.3. Salt tolerance test

GYEA media were prepared in duplicate at various salt concentrations at 2%, 3%, 4%, 5%, 6%, 7%, 7.5%, 8%, 9%, 10%, 12%, 12.5% and 15%, determined from previous studies. After inoculation, culture plates were allowed to dry before incubation at 28°C for 14 days. The culture plates were compared to each other and the presence of growth was scored positive.

3.14.3.4. Catalase reaction test (Gordon and Mihm, 1962)

Three day old cultures of the test representative type strains were prepared. A drop of 20% (v/v) hydrogen peroxide (H_2O_2) solution was placed on the microscope slides and subsequently, a loop full of cultures grown on GYEA medium was transferred to the drop of 20% (v/v) H_2O_2 solution. Positive result was noted when intensive bubbling occurred immediately or within 5 minutes.

3.14.3.5. Oxidase test (Lányi, 1987)

Fresh biomass of the test isolates and the type strain representatives was recovered using a sterile plastic loop (to prevent false positive result) and thinly smeared onto filter paper strip placed on a microscope slide. Subsequently, the specimen was impregnated with oxidase test reagent that contained NNN'N'-Tetramethyl-p-phenylenediamine dihydrochloride.

3.14.3.6. Hydrogen sulphide (H_2S) production, nitrate and nitrite reduction tests

The capacity of the test strains to produce hydrogen sulphide during growth was detected using lead acetate paper strips (Appendix 7b). 5 µl of test strains in suspension (in 5.0 McFarland densities; Appendix 7b) were inoculated into 3 ml of nitrate broth (Appendix 7a) in bijou tubes. Lead acetate paper strips hanging at the neck of the tube were examined after 14 days of incubation at 28°C. Positive score was given when lead acetate paper strips blackened. Lead acetate was converted to lead sulphide by hydrogen sulphide and therefore resulted in the blackening of the lead acetate paper. After hydrogen sulphide production test, nitrate and nitrite reduction tests were carried out using the same cultured broth. Reagent A containing sulfanilic acid and Reagent B made up of α-naphthylamine (Appendix 7a) were added to the cultures. The development of red colour indicates the presence of nitrite and positive for nitrate reductase activity. Cultures with no colour change were then had zinc dust added to them to examine of this lead to a colour change. If a red colour developed, it indicated nitrate and nitrite reductase negative while with the absence of colour change, the reactions would be scored positive for nitrate and nitrite reductase due to the addition of zinc dust as it showed that nitrate has been reduced to nitrogen gas.

3.14.3.7. Methyl red (MP) and Voges-Proskauer (VP) tests (Lányi, 1987)

An aliquot of 5 µl of bacterial suspensions (in 5.0 McFarland densities) were inoculated in 3ml of MR-VP test broth (Appendix 7a) in bijou tubes. Cultures were examined after 4 days of incubation at 37°C. Aliquots of 500 µl of the cultures were transferred to test tubes for analysis. Approximately 3-4 drops of methyl red reagent (Appendix 7b) were added to examine for colour change. Instant changed of colour to red indicated positive results in which bacteria can produce acid to maintain the pH below 4.5 for several days. The same remaining cultures were then used to carry out Voges-Proskauer test. A volume of 0.6 ml of Reagent A (methanolic α-Naphthol) was first added, followed by 0.2 ml Reagent B (potassium hydroxide) (Appendix 7b). The cultures were shaken and left for 15 to 60 minutes before examination. The development of red colour indicated positive results. No colour reaction, faint pink or copper colours indicate negative results.

3.14.3.8. Indole production test (Lányi, 1987)

0.2 ml of Kovács's reagent (Appendix 7b) was added to 3 ml bacto peptone cultures (Appendix 7a) after 3 days of incubation at 28°C. The bijou tubes were left unshaken for the development of red colour. Deep red colour in the reagent layer showed positive results and negative when no colour changed.

3.14.3.9. Lysozyme resistance test

An aliquot of 5 µl of bacterial suspension (in 5.0 McFarland densities) were inoculated into 3 ml glycerol broth containing 0.005% (w/v) lysozyme in bijou tubes (Appendix 7a). A negative control without lysozyme was set up. Inoculated tubes were incubated at 28°C for 14 days. Strains were scored positive when growth was greater or equal to that in the control glycerol broth and negative when no growth was observed.

3.14.3.10. Degradation tests

A total of 22 degradation analyses was carried out using the following substrates; 0.1% adenine, 0.1% aesculin, 1% allantoin, 0.1% arbutin, 0.1% casein, 1% cellulose, 0.3% DNA, 0.3% elastin, 0.4% gelatin, 0.5% guanine, 0.4% hypoxanthine, 0.3% RNA, 0.1% starch, 1% tributyrin, 0.5% L-tyrosine, 1% Tween 20, 40 and 60, 0.2% urea, 0.5%

uric acid, 0.4% xanthine and 0.4% xylan, with GYEA medium as the basal medium (Appendix 7a). An aliquot of 1 µl from each test strain suspension prepared according to section 3.15.1 was used to inoculate each respective medium (in duplicate) and incubated at 28°C. An aliquot of 5 µl of test strain suspension was used for allantoin and urea degradation tests. The periods of incubation and methods of analysis were summarised in Appendix 9.

3.14.3.11. Acid production tests

An aliquot of 1 µl of each test strain suspension was inoculated onto the basal medium of Hugh and Leifson (1953) (Appendix 7a) in duplicate which was supplemented with 1% (w/v) carbohydrate solutions (total of 27 carbon sources; Appendix 10) and bromothymol blue as pH indicator. Inoculated plates were incubated upright, without inverting the culture plates at 28°C for 14 days. Plates were checked after 7 and 14 days. Changes of colour from blue to orange-yellow were scored as positive while no colour change indicated a negative score.

3.14.3.12. Utilisation of carbohydrates and amino acids as sole carbon, energy and nitrogen sources

Test strains were examined for their ability to use 58 carbon compounds at 1%, 34 carbon sources at 0.1%, as sole carbon sources for energy and growth (Appendix 10), and their ability to utilise 31 amino acids at 0.1% (w/v) as nitrogen sources by using Stevenson's medium (Appendix 7a). The medium plates supplemented with 1% and 0.1% (w/v) D (+)-glucose, respectively were used as positive controls while the carbon-free Stevenson's medium was served as a negative control. Inoculated plates with 1 µl of test strain suspension were cultured at 28°C. Growth cultures were examined after 7, 14 and 21 days. Test strains were scored positive if growth on the test plate was greater than that on the negative control plate and negative if growth was equal to or less than that on the negative control plate.

3.14.3.13. Detection of bacterial enzymatic reactions

A total of 19 enzymatic tests were carried out using API ZYM (biomérieux) strips. Each test strain was prepared to a suspension with 5.0 McFarland densities. The incubation box was first prepared by distributing 5 ml of sterile distilled water to create

a humid atmosphere. After inoculation, each test strip in the incubation box was incubated at 37°C in the dark for 4.5 hours. Subsequently, the analysis and interpretation were carried out according to the manufacturer's instructions.

3.15. Chemotaxonomic characterisation:

3.15.1. Preparations of dried biomass

The 19 novel representative isolates selected for phenotypic characterisation and the related type strains of *Dermacoccus nishinomiyaensis* DSM 20448, *Dietzia maris* DSM 43672, *Leifsonia soli* DSM 23871, *Rhodococcus rhodochrous* DSM 43241 were cultured on GYEA media and incubated for two to three days at 28°C until the visible growth of single colonies can be observed. After incubation, biomass were taken using a sterile loop and transferred to each of the four 250 ml conical flasks containing 100 ml of sterile GYE broth (Appendix 7a). The inoculated conical flasks, which were fitted with sterile cotton wool plugs, were incubated with reciprocal shaking at 200 rpm in an orbital incubator (New Brunswick Scientific Innova 44) for 5 days at 28°C. After incubation, the cultures were transferred to sterile 50 ml centrifuge tubes and centrifuged at 6,000 rpm for 20 minutes. The supernatant was discarded and biomass pellet was washed with sterile distilled water and this process was repeated twice more. Harvested biomass was stored at -80°C freezer overnight and then lyophilised in a vacuum freeze drier (CHRiST ALPHA 1-2 LD plus) at -51°C for 2 days until the biomass was completely dried. Dried biomass samples were then stored in the dark at room temperature.

3.15.2. Acid methanolysis

Dried biomass of *ca*. 25 mg of each 19 representative isolates, alongside the marker strains of *D. nishinomiyaensis* DSM 20448, *D. maris* DSM 43672, *L. soli* DSM 23871 and *R. rhodochrous* N54^{T} were weighed out in tall Teflon-lined screw-capped tube. To each of the biomass sample, 2 ml of 1.5% v/v of methanolic sulphuric acid (Appendix 7b) was added and the samples were incubated at 50°C overnight to allow trans-esterification. After an overnight incubation, 1ml of sterile distilled water and 3ml of hexane were added to the acid esterified biomass samples before they were mixed by shaking vigorously. The mixed solution was then allowed to stand to 'break' the emulsion. The upper solvent layer of hexane was transferred using a glass Pasteur

pipette to a clean tube and the remaining sample was vigorously shaken with a further 3 ml of hexane. The step with 3ml of hexane added was repeated once more and the upper hexane layers were pooled with the first extract. A volume (9 ml) of sterile distilled water was added to the pooled hexane fractions and it was gently shaken to wash. Subsequently, the phases were allowed to stand and separate and the washed hexane fraction was transferred to a clean dry tube containing a spatula full of anhydrous sodium sulphate as a drying agent. The hexane fractions that contained the fatty acids were concentrated under nitrogen stream to approximately 1ml and it was transferred to a small screw cap storage vial and further concentrated down to approximately 25 µl. The extracts were stored at -20°C prior to GC/MS analysis.

3.15.3. Alkaline methanolysis

The whole-organism alkaline methanolysis procedure was modified from that described by Minnikin (1988, 1993). Dried biomass (*ca*. 50 mg) of each test strain was suspended in 2ml of 5% v/v aqueous tetrabutylammonium hydroxide diluted from a 40% commercial solution (Sigma) held in 8.5 ml Corning tubes. The mixtures were heated overnight in (TECHNE sample concentrator Dri-Block DB3) at 100 °C and then cooled to room temperature prior to centrifugation at 2000 rpm for 5 minutes. The supernatants were transferred to clean tubes containing 2 ml of dichloromethane and 25 µl of iodomethane and the resultant preparations were shaken on a tumble shaker (Stuart Rotator SB2) for 30 minutes. The preparations were then centrifuged and the upper aqueous layers were discarded. The lower organic layers that contained the methyl ester derivatives were washed with 1ml of a 10% aqueous HCl solution followed with 1ml of sterile distilled water. The washing step was repeated twice before transferring the lower organic layers to a clean small glass vials for immediate evaporation to dryness under a stream of nitrogen gas at room temperature to prevent oxidation of the extracts and the dried extracts were stored at 4°C prior to thin-layer-chromatography (TLC) analysis.

3.15.4. Extraction and detection of polar lipids

Polar lipids were extracted from the test strains using the small-scale integrated procedure of Minnikin *et al*. (1984). Dried biomass of *ca*. 50 mg was weighed out in 8.5 ml Corning glass tubes, fitted with Teflon-lined screw caps, containing 2 ml aqueous methanol (Appendix 4b) and 1 ml of petroleum ether (b.p. 60 – 80°C). Each tube was

tightly sealed before mixing the contents for 15 minutes on a tumbler shaker (Stuart Rotator SB2). The preparations were the centrifuged at 3,500 rpm for 5 minutes and the upper organic phase, which contained the isoprenoid quinones were stored in a small glass vials. Subsequent to the removal of the isoprenoid quinones in the upper organic phase, the lower layer containing polar lipids and the cell debris were heated in a boiling bath (120°C) for 30 minutes. After the tubes were cooled to room temperature, 2.3 ml of chloroform-methanol-0.3% aqueous sodium chloride (90:100:30, v/v) was added and the contents were mixed for 1 hour on a tumble shaker (Stuart Rotator SB2). The samples were centrifuged at 3,500 rpm for 15 minutes and the supernatants were transferred to clean 8.5 ml Corning glass tubes. A further 0.75 ml of chloroform-methanol-0.3% aqueous sodium chloride (50:100:40, v/v) was added to the remaining biomass, the contents was mixed for 30 minutes, centrifuged as before and the resultant supernatant was pooled with the first extract. The extraction using chloroform-methanol-0.3% aqueous sodium chloride (50:100:40, v/v) was repeated again. 1.3 ml of chloroform and 1.3 ml of 0.3% w/v aqueous sodium chloride were added to the pooled supernatants and the resultant preparations were thoroughly mixed by repeated inversion for 5 minutes before centrifugation at 3,500 rpm for 10 minutes. The upper layer was discarded and the lower layer, which contained the polar lipids were transferred to 3 ml glass vials and evaporated under nitrogen stream prior to storing at 4°C until required for two-dimensional-TLC analysis.

3.15.5. One-dimensional Thin-Layer-Chromatography analysis (1D-TLC)

The mycolic acid methyl esters extracts (MAMEs) were separated by one-dimensional ascending thin-layer-chromatography (TLC), as described by O'Donnell *et al*. (1982). Aluminium backed silica gel TLC sheets (20 cm × 20 cm; Merck no. 5554, Silica gel 60 F_{254}) were cut down to 10 cm × 20 cm in size. The extracts of each sample were re-dissolved in 50 µl of dichloromethane and a small volume of *ca*. 5 µl was spotted little by little onto a line marked 1cm from the base of each sheet to prevent excessive spreading using a glass capillary pipette. Each TLC plate was spotted with 7 methanolysate samples with 1 cm distance apart from each other, leaving 2 cm distance at both ends. The methanolysate samples were run alongside with the corresponding marker strains in order for comparison to be made. The aluminium plates loaded with the sample extracts were developed in glass tank containing light petroleum ether (b.p. 60 – 80°C) and acetone at a ratio of 92:8 (v/v) as the solvent system. The plates were

removed when the solvent front reached the 1 cm line marked before the top and the chromatograms were air dried before a second time of development in the same direction. After the second run, the chromatograms were soaked with 5% of ethanolic molybdophosphoric acid (Appendix 4b) and were immediately dried in an oven at 180°C for 5 minutes. The dark blue/black spots revealed on a pale green background were the positions of the separated MAMES. The chromatograms were scanned immediately to capture the visible MAMES spots. The chromatograms were scanned immediately to capture the visible mycolic acid spots. Mycolic acids were determined by calculating the R_f value (distance of spot travelled/distance of solvent travelled) and compared to the neighbouring type strains.

3.15.6. Two-dimensional Thin-Layer-Chromatography analysis (2D-TLC)

Two-dimensional TLC as described by Minnikin *et al*. (1984) was carried out to partial characterise the dried polar lipid extracts. The polar lipids extracts were re-dissolved in 200 µl of chloroform-methanol (2:1, v/v) mixed solvent, and 20 µl of aliquots were from each extract was applied to the bottom left corner of a silica gel aluminium plate in the size of 10 cm × 10 cm cut out from 20 cm × 20 cm sheets (Merck no. 5554, Silica gel 60 F_{254}), 1 cm from the left side and up from the base. The solvent mixtures were prepared fresh and allowed to equilibrate in the glass tanks lined with Whatman filter papers (185 mm) for 1 hour prior to use. The plates were developed in the first direction using the solvent system composed of chloroform-methanol-water (65:25:3.8, v/v), air dried for 15 minutes and developed in the second direction, at the right angle to the first, using chloroform-acetic acid-methanol-water (40:7.5:6:1.8, v/v) as the solvent system. The chromatograms were further air-dried for 15 minutes and were stained with 5% of ethanolic molybdophosphoric acid. The plates were then charred at 150°C for 15 minutes to detect all the polar lipids in which they appeared as dark blue/black spots on a pale green background. The chromatograms were scanned immediately to capture the visible polar lipids spots. The polar lipids were identified and determined by comparing to the reported neighbouring type strains.

Chapter 4 Detection and Profile Analysis of Bacterial and Archaeal Communities

4.1. Background

The distribution of microbial populations in Lake Suigetsu has been explored. Kondo *et al*. (2006) investigated the depth distribution of sulphate-reducing bacteria (SRB) in the water column of Lake Suigetsu and they showed the highest diversity and abundance of SRB communities at the interface of oxic-anoxic zone. The obligate anaerobic SRB are known to play important roles in biogeochemical cycling of sulphur and mineralisation of organic matter in environments lacking of oxygen (Jørgensen 1982; Kondo *et al*., 2008). Therefore, the understanding of the SRB functions in this anaerobic ecosystem is important for the comprehension of ecological and biogeochemical processes within the water column.

Further study reported that the dominating green and colourless sulphur bacteria in the lake's chemocline may also contribute to the sulphur and carbon cycling (Kondo *et al*., 2009). In addition, Bacteroidetes that were abundant throughout the water column were believed to have roles in fermentative metabolism and degradation of organic matter in Lake Suigetsu (Kondo *et al*., 2009). Besides important studies of bacterioplankton populations that could expand the understandings of biogeochemical processes and microbial functions in the limnic ecosystems (Gugliandolo *et al*., 2011; Kondo *et al*., 2009; Lehours *et al*., 2005; Øvreås *et al*., 1997), picophytoplanktons communities on the halocline of Lake Suigetsu were also being explored through pigments and HPLC analyses. This has revealed picocyanobacteria have been found to be the primary producers in the halocline of Lake Suigetsu (Okada *et al*., 2007), which echoes the photosynthetic communities seen in other lakes and marine environments (Callieri and Stockner 2002; Malinsky-Rushansky *et al*., 2002; Stockner, 1988).

Former studies mainly focused on the vertical microbial profile in the lake water column (Kondo *et al*., 2006; Kondo *et al*., 2009; Okada *et al*., 2007). Although a previous study looking at the bottom surface sediment by Kondo and Butani (2007) to compare SRB compositions with the lake waters, the microbial diversity remains largely unexplored in the deeper sediment as only the top 4 cm of the surface sediment

was examined. In their study, they found that the microbial communities in the lake water column were significantly different to those of the bottom surface sediments. Microbial roles in biogeochemical processes and their responses to environmental change in deep sediments are therefore still poorly understood (Borrel *et al*., 2012; Shade *et al*., 2012; Griffiths and Philippot, 2013).

This study represents the first microbial study of the deeper sediment core of Lake Suigetsu. This is the preliminary attempt to explore the microbial diversity in the deeper sediments of Lake Suigetsu and compare the structure of microbial community between shallow and deep sediments. The aim is to elucidate if the microbial community structures are randomly distributed, depth-related or affected by other environmental variables. In addition, Lake Suigetsu has a very precise event stratigraphy owing to its annually laminated varves and so offers an opportunity to explore and discover potential paleoenvironmental indicators for past climate change. We hypothesise that the undisturbed microbial communities correlate to the past conditions of an earlier biosphere (Gugliandolo *et al*., 2011; Lehours *et al*., 2007). The outcome of microbial data can then be used to relate and compare with the analysed pollen data and constructed biome for past climate by Nakagawa *et al*. (2005) to identify possible molecular biomarkers.

4.2. Experimental strategy:

Sedimentary records at every 4 m down the sediment core of Lake Suigetsu were taken. The sampling points were matched based on age (BP) with the biome records of Lake Mikata (Gotanda *et al*., 2002). Fossil DNA was extracted and specific groups of taxa were targeted using group-specific primers sets and PCR while DGGE was used to study the bacterial communities. The relationship between environmental variables and bacterial communities from different biome zones was examined to identify potential biomarkers for past climate.

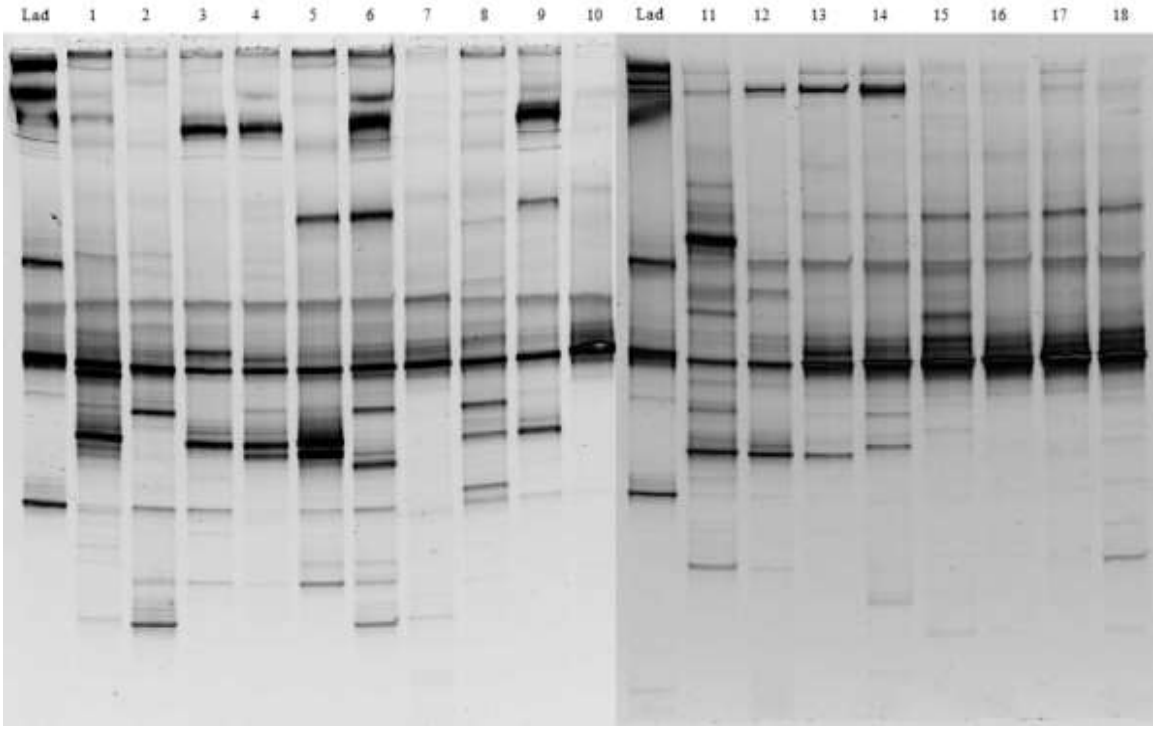
4.3. Results

4.3.1. DGGE analysis of microbial communities

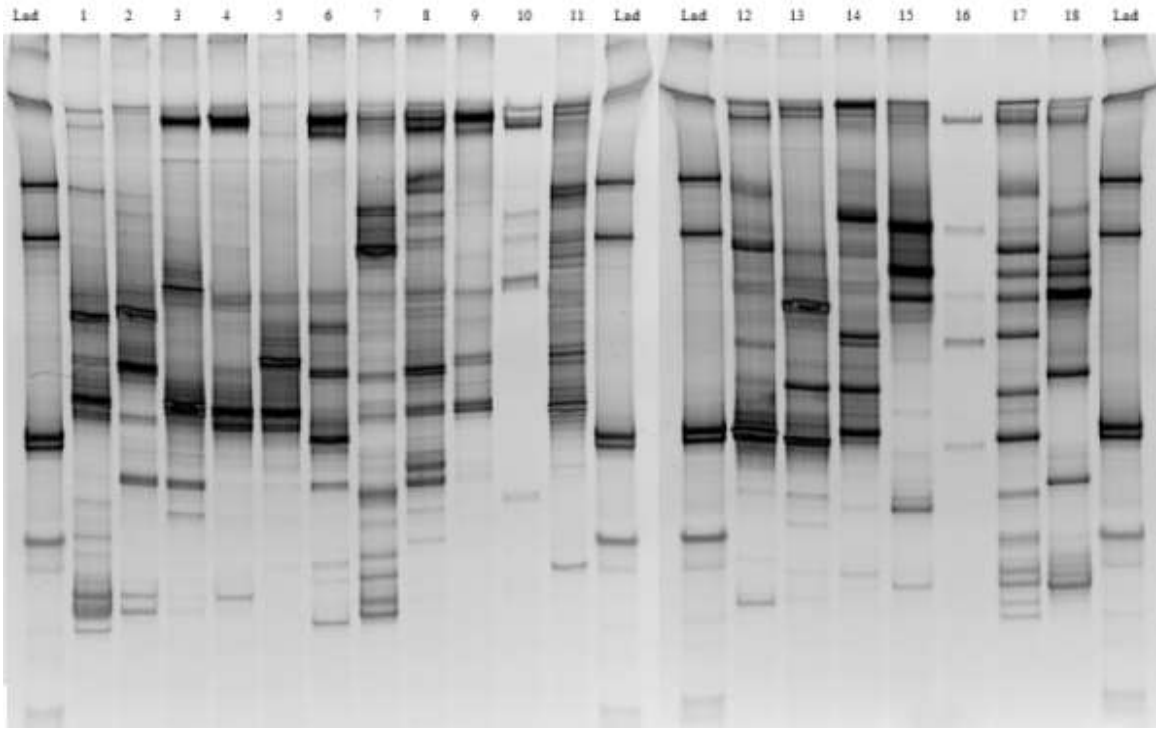
The reproducible PCR products of Bacteria, *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* as well as *Archaea*

were subjected to DGGE analysis and DGGE fingerprint profiles were produced (Figure 4.1a to 4.1g). The DGGE banding patterns of each community profile appeared to be greatly different from each other. Each DGGE band was presumed as a single taxon and as a measure of species richness. So, the higher the number of DGGE bands in the DGGE lane, the higher the number of taxa in the particular sediment.

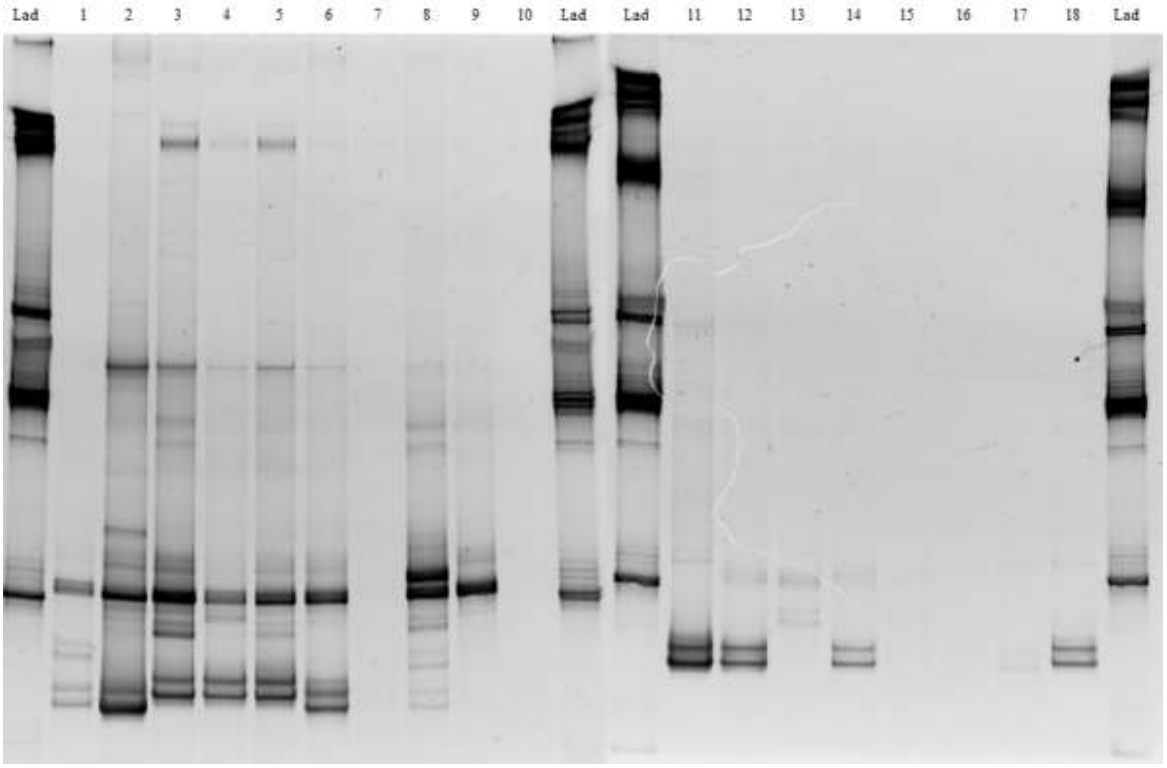
a) *Bacteria*



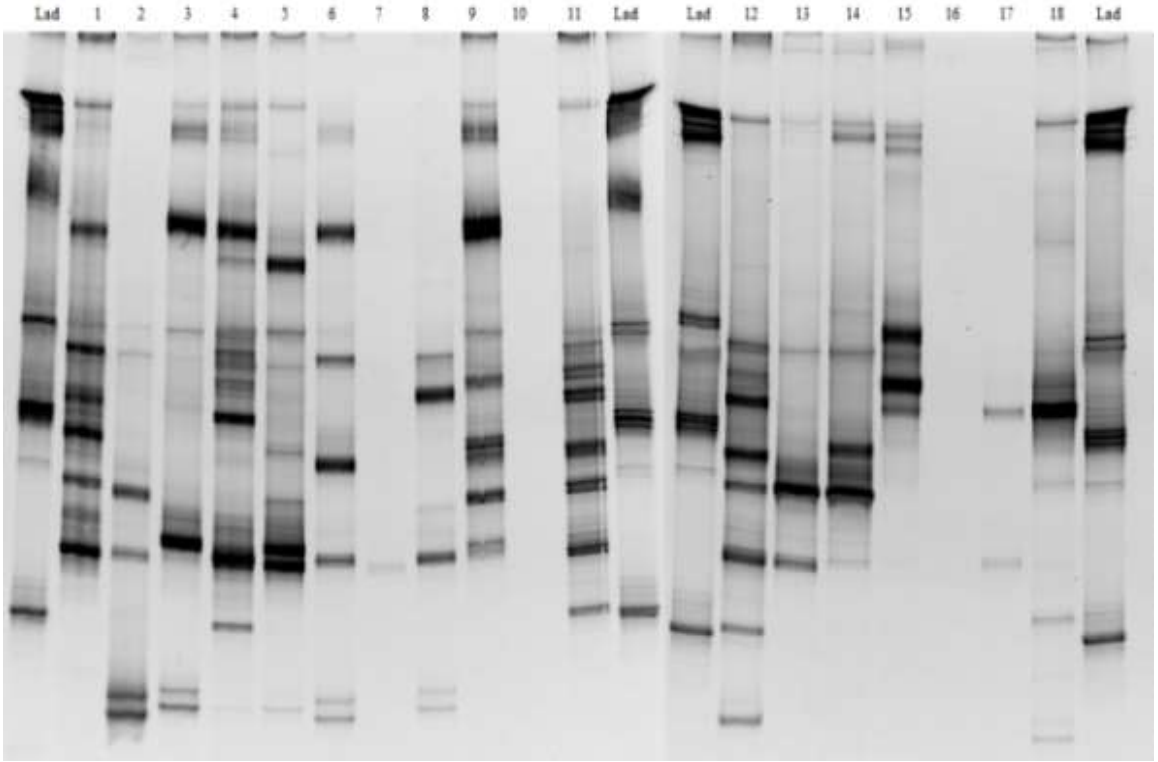
b) *Acidobacteria*



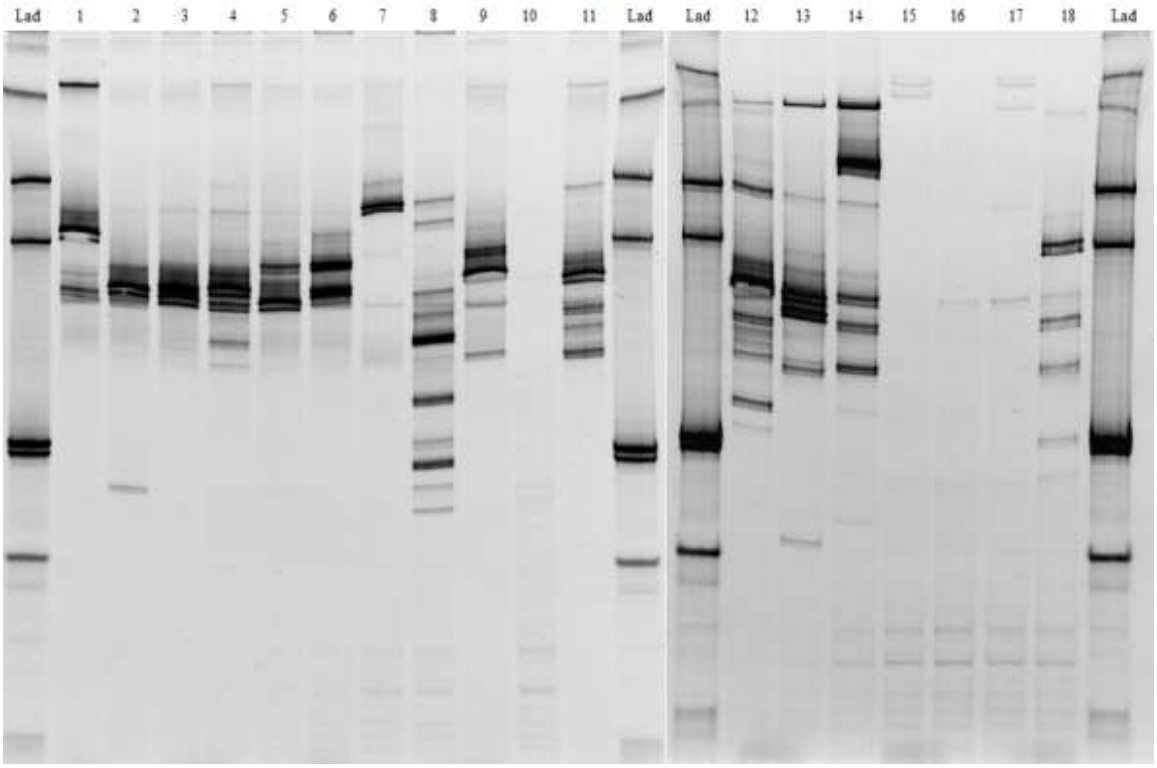
c) *Actinobacteria*



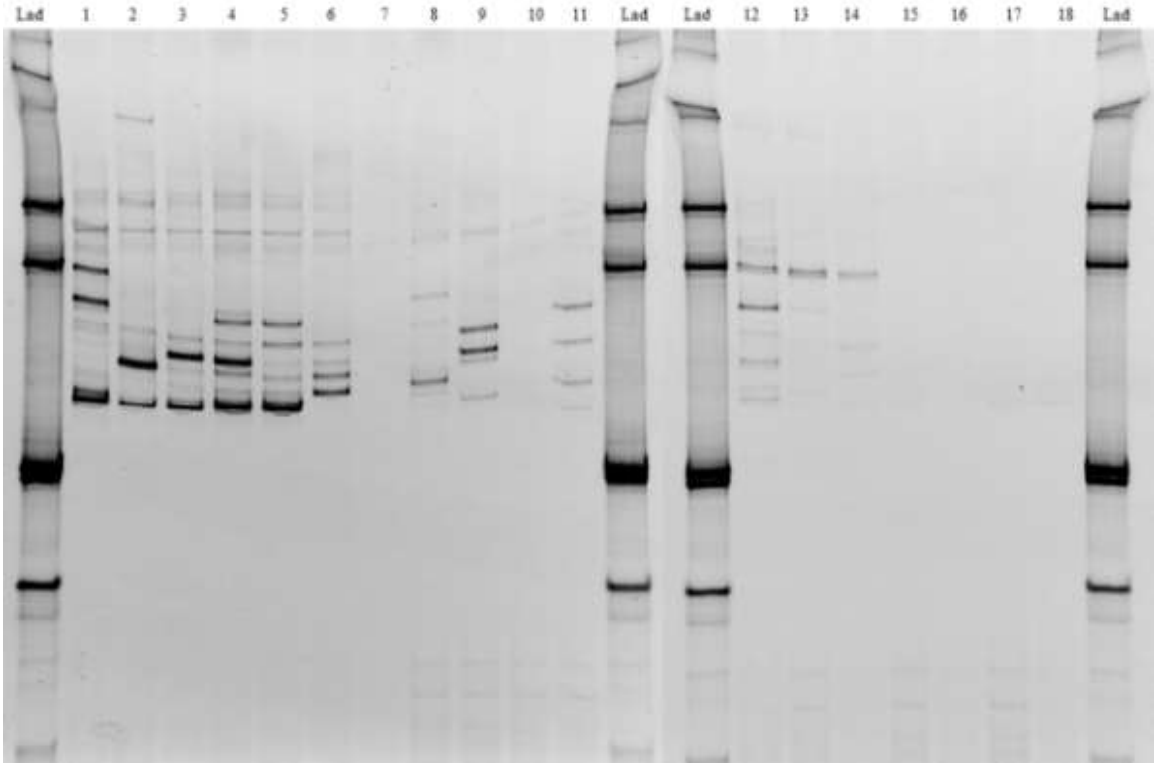
d) *Alphaproteobacteria*



e) *Betaproteobacteria*



f) *Gammaproteobacteria*



g) *Archaea*

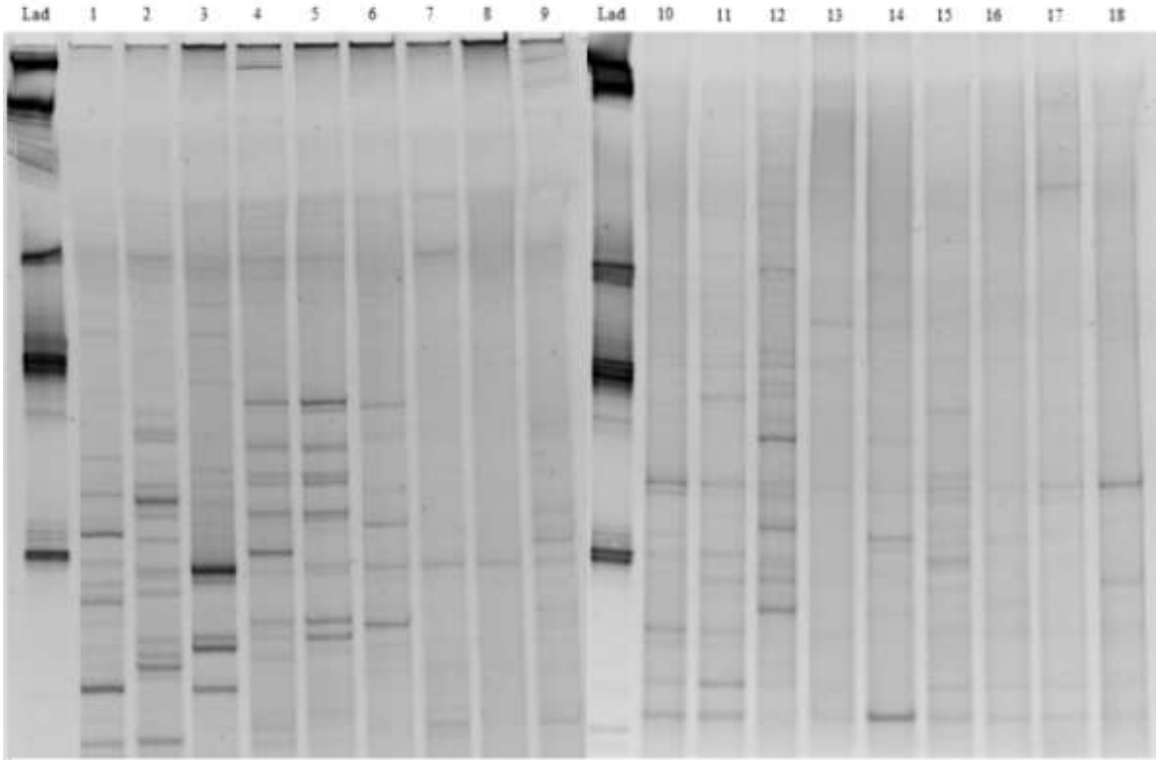


Figure 4.1: DGGE profile of 16S rDNA genes based on each community DNA analysis from depth 148.55 cm to 7247.69 cm. a) Bacteria, b) *Acidobacteria*, c) *Actinobacteria*, d) *Alphaproteobacteria*, e) *Betaproteobacteria*, f) *Gammaproteobacteria*, g) *Archaea*. Lad = ladder, 1= A (N) 01; 2= A (N) 03; 3= B (N) 05; 4=B (N) 07; 5= B (N) 11; 6= A (S) 13; 7= A (N) 16; 8= B (N) 17; 9= B (N) 19; 10= B (N) 21, 11= A (N) 24; 12= A (N) 28; 13= A (S) 30; 14= A (S) 32; 15= A (S) 35; 16= A (N) 39; 17= A (N) 43; 18= A (N) 46.

Table 4.1: Characterisation of prokaryotic community profiles based on species richness in Lake Suigetsu with increasing sediment depth

Biome	Core	Age (BP)	Depth (cm)	Rr (Species richness)						
				Bac.	***Arch.***	***Acido.***	***Acti.***	***α-pro.***	***β-pro.***	***γ-pro.***
WAMX	A(N) 01	567	148.55	29	17	24	6	19	10	12
	A(N) 03	4124	599.53	26	18	22	12	7	7	8
	B(N) 05	6860	932.44	22	19	24	13	10	7	7
	B(N) 07	10911	1363.76	25	21	21	8	23	13	11
COMX /TEDE	B(N) 11	22502	2150.45	27	15	29	11	16	10	9
TEDE	A(S) 13	28911	2510.00	28	8	25	9	10	7	6
	A(N) 16	34945	2930.22	15	7	35	0	1	7	0
	B(N) 17	41635	3359.29	27	2	30	12	5	14	8
	B(N) 19	48376	3761.25	19	12	14	4	15	9	7
UNKNOWN	B(N) 21	60354	4197.10	13	11	8	0	0	4	3
	A(N) 24	74328	4549.76	26	13	35	7	21	9	7
	A(N)28	88842	4998.20	16	20	22	4	14	14	11
	A(S) 30	101916	5400.68	16	6	24	4	8	9	7
	A(S) 32	112998	5799.90	19	10	25	3	14	15	3
	A(S) 35	121274	6199.88	17	12	22	0	12	5	3
	A(N) 39	128993	6549.74	12	6	5	0	0	3	0
	A(N) 43	136166	6946.65	16	8	17	1	2	7	5
	A(N) 46	143019	7247.69	20	6	16	2	10	10	1
			Total	373	211	398	96	187	160	108

Core no. with A (N) 01 is the label given; A represents the borehole, N represents the direction of the core- where N (North) indicates the first 100 cm of the core and S (South) indicates the bottom 100 cm of the core. The number after the N or S represents the core number and composite depth is calculated from the top core. Rr, the community richness indicates the numbers of taxa in each DGGE lane. Biome zones indicated by WAMX = warm mixed forest, TEDE = temperate deciduous forest and COMX = cool mixed forest.

Table 4.1 shows that in general, the distribution of the number of taxa for each community profile was uneven throughout the sediment cores. The species richness of each community's DGGE profile was higher on the upper cores compared to the deeper sediments. Among the DGGE profiles of the group specific bacteria, *Acidobacteria* community has the highest number of species richness (398) throughout the sedimentary records of Lake Suigetsu whereas *Actinobacteria* community appeared to be the least (96).

Table 4.2: Characterisation of prokaryotic community profiles based on diversity in Lake Suigetsu with increasing sediment depth

Biome	Core	Age (BP)	Depth (cm)	*H'* (Shannon-Wiener Index)						
				Bac.	***Arch.***	***Acido.***	***Acti.***	***α-pro.***	***β-pro.***	***γ-pro.***
WAMX	A(N) 01	567	148.55	2.791	2.297	2.720	1.523	2.549	2.065	1.671
	A(N) 03	4124	599.53	2.693	2.448	2.555	1.733	1.320	1.490	1.336
	B(N) 05	6860	932.44	2.706	1.841	2.531	2.188	1.596	1.397	1.227
	B(N) 07	10911	1363.76	2.678	2.435	2.158	1.560	2.555	1.990	1.727
COMX /TEDE	B(N) 11	22502	2150.45	2.702	2.688	2.832	1.825	2.129	1.798	1.383
TEDE	A(S) 13	28911	2510.00	3.093	1.534	2.461	1.670	1.732	1.159	1.316
	A(N) 16	34945	2930.22	1.897	1.759	3.155	0	0	1.093	0
	B(N) 17	41635	3359.29	2.726	0.691	2.935	1.491	1.092	2.173	1.553
	B(N) 19	48376	3761.25	2.605	2.469	1.964	0.375	2.197	1.755	1.383
UNKNOWN	B(N) 21	60354	4197.10	1.681	1.602	1.760	0	0	1.271	1.074
	A(N) 24	74328	4549.76	2.869	2.265	3.229	0.868	2.471	2.007	1.482
	A(N)28	88842	4998.20	2.502	2.514	2.693	0.905	2.248	2.265	1.815
	A(S) 30	101916	5400.68	2.35	0.676	2.810	1.222	1.278	1.797	1.216
	A(S) 32	112998	5799.90	2.401	1.856	2.576	0.831	2.193	2.063	0.802
	A(S) 35	121274	6199.88	2.127	2.324	2.635	0	1.895	1.540	0.520
	A(N) 39	128993	6549.74	1.694	1.732	1.472	0	0	0.994	0
	A(N) 43	136166	6946.65	1.888	1.413	2.645	0	0.568	1.817	0.568
	A(N) 46	143019	7247.69	2.244	0.839	2.419	0.693	1.615	1.793	0
			Total	2.425	1.855	2.529	0.938	1.524	1.692	1.06

Core no. with A (N) 01 is the label given; A represents the borehole, N represents the direction of the core- where N (North) indicates the first 100 cm of the core and S (South) indicates the bottom 100 cm of the core. The number after the N or S represents the core number and composite depth is calculated from the top core. H' represents the Shannon diversity indices for eubacteria and *Achaea* profiles. Biome zones indicated by WAMX = warm mixed forest, TEDE = temperate deciduous forest and COMX = cool mixed forest.

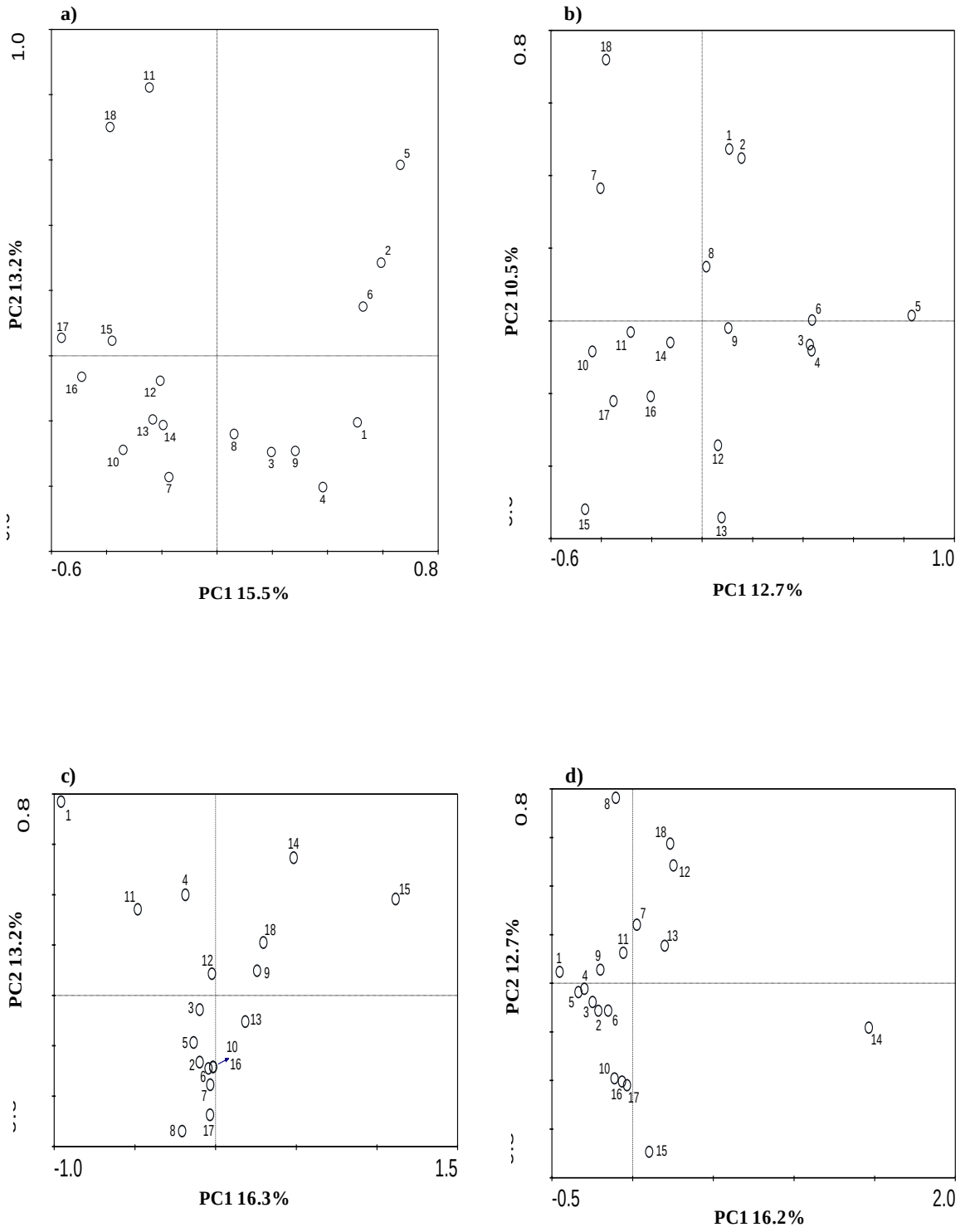
Shannon diversity index (*H*') that takes account of both species richness and evenness (Kennedy and Smith, 1995) was also calculated based on the DGGE banding patterns of each community profile. The higher the *H*' value the greater the diversity of the bacterial community. The Shannon diversity of each community profile was overall higher on the upper sediments and lower in the deepest cores (Table 4.2).

4.3.2. PCA analysis

Principle component analysis (PCA) was employed given that a large set of variables was used. PCA simplifies complex data sets to identify the most important gradients explaining the variance within the data at maximum visibility (Hammer *et al*., 2001). In PCA, the first principle component (PC1) axis primarily accounts for the largest eigenvalue and explains the greatest variance in the data set while the second component (PC2) axis accounts for the second greatest (Hammer *et al*., 2001). In Figure

4.2a of bacterial community, most of the sediment samples separated along PC1 axis which explained 15.5% of the total variance in the communities while PC2 accounted for 13.2%. Although bacterial community on the upper cores appeared to be distributed on the positive axis of PC1 which was separated from the community in the deepest cores that fell on the negative axis of PC1, such low total variance did not indicate strong relationship between depth and bacterial community.

The variance of *Acidobacteria* profile that was explained by PC1 and PC2 are 12.7% and 10.5%, respectively (Figure 4.2b). This also indicated a weak support of depth effect on the community of *Acidobacteria*. For the profile of *Alphaproteobacteria*, both principal components did not account for a huge total variance which is less than 30% (Figure 4.2c). Therefore, this showed no clear evidence of depth-related distribution for the *Alphaproteobacteria* community, likewise the PCA of the *Betaproteobacteria* profile (Figure 4.2d). For *Gammaproteobacteria*, PC1 showed a slightly higher total variance of 18.7% while PC2 accounted for 12.4% of the remaining variance (Figure 4.2e). In addition, the sediment samples of the upper cores were distributed towards the negative axis of PC1 separated from the communities in the deeper sediments. This may indicate the effect of depth exerted on the *Gammaproteobacteria* communities. Furthermore, from the PCA profile of *Actinobacteria*, both the PC1 and PC2 showed a total variance of about 40% which indicates a strong correlation of depth effect that is likely to affect the distribution patterns of *Actinobacteria* populations (Figure 4.2f). The PCA profile of domain *Archaea*, unlike the domain bacteria, did not show evident correlation with depth as the total variance accounted by both PC1 (13.6%) and PC2 (12.9%) was low (Figure 4.2g).


a)
1.0
PC2 13.2%
-0.6
0.8
PC1 15.5%
b)
0.8
PC2 10.5%
-0.6
1.0
PC1 12.7%
c)
0.8
PC2 13.2%
-1.0
1.5
PC1 16.3%
d)
0.8
PC2 12.7%
-0.5
2.0
PC1 16.2%

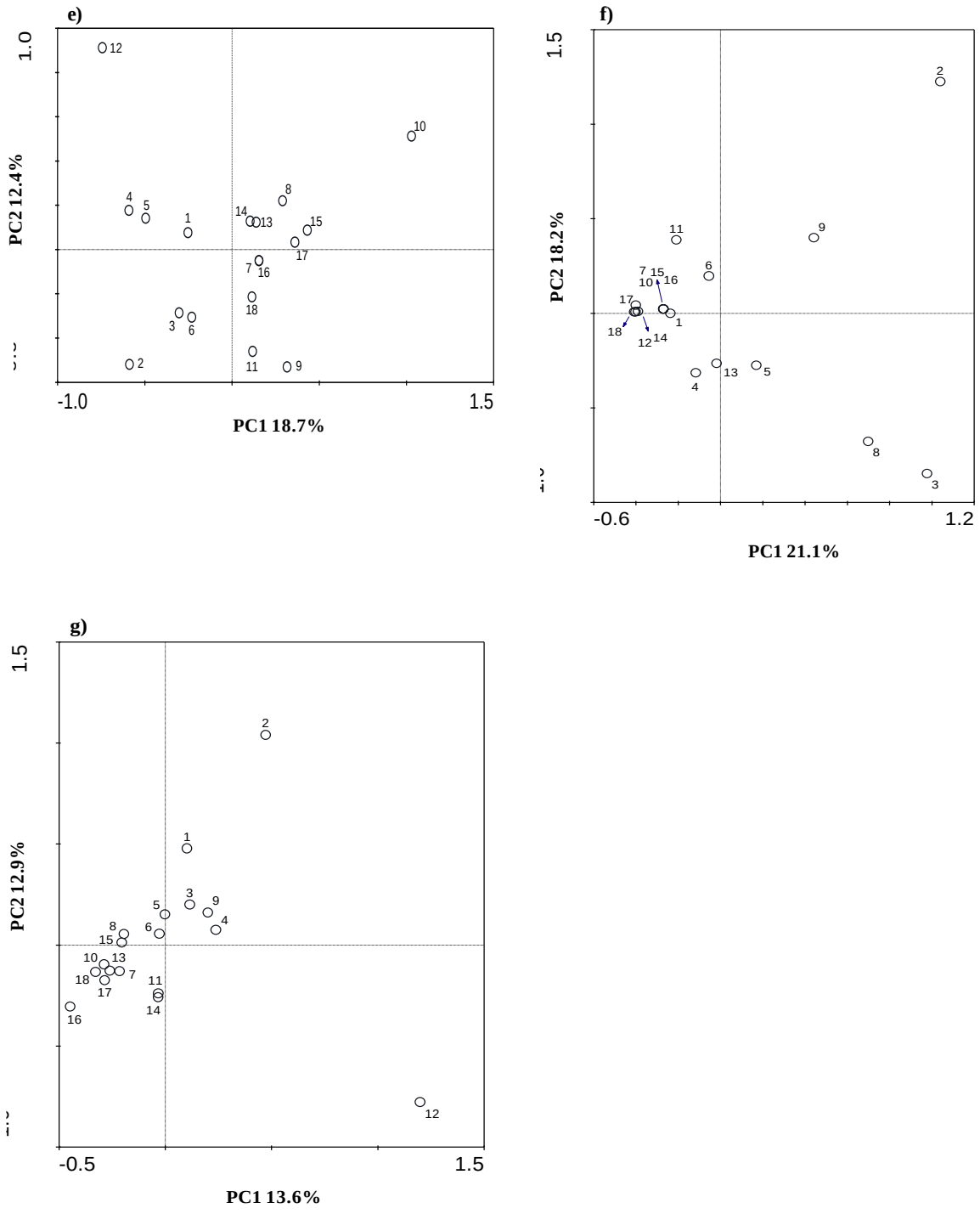


Figure 4.2: PCA analysis of each community profile. a) bacteria, b) *Acidobacteria*, c) *Alphaproteobacteria*, d) *Betaproteobacteria*, e) *Gammaproteobacteria*, f) *Actinobacteria*, g) *Achaea*. Sediment sample 1 = A (N) 01; 2 = A (N) 03; 3 = B (N) 05; 4 = B (N) 07; 5 = B (N) 11; 6 = A (S) 13; 7 = A (N) 16; 8 = B (N) 17; 9 = B (N) 19; 10 = B (N) 21; 11 = A (N) 24; 12 = A (N) 28; 13 = A (S) 30; 14 = A (S) 32; 15 = A (S) 35; 16 = A (N) 39; 17 = A (N) 43; 18 = A (N) 46.

4.3.3. Raup-Crick Similarity index

Raup and Crick Similarity Index analysis (Table 4.3) was also calculated for each community profile to identify the similarities between bacterial community structures whether they were significantly higher or lower than would be observed by chance (Baxter and Cummings, 2006; Rowan *et al*., 2003). Raup and Crick similarity index (S_{RC}) with $\geq$ 0.95 (95%) indicate significant similarities between bacterial community profiles and significant dissimilarities when $S_{RC} \leq 0.05$. S_{RC} values that fell within 0.05 and 0.95 are taken to represent similarities that occurred by chance.

Of the top five sediment samples 1 to 5 within the bacterial community, one sample pair (3 and 4) appeared to be significantly similar to one another, showing 97% of similarities while the bacterial communities in the middle cores (sediment samples 6 to 10) have three that were significantly similar between each other. In the deepest sediments (11-18), 18 were found to be significantly similar to each other but were quite different from the communities in the upper cores. The communities of *Acidobacteria* (Table 4.4), *Actinobacteria* (Table 4.5), *Alphaproteobacteria* (Table 4.6) and *Archaea* (Table 4.9) also showed similar trends. In contrast, the *Betaproteobacteria* communities (Table 4.7) appeared to be significantly similar to each other in the upper cores (sediment samples 1 to 5) and only two sample pairs in the middle cores (5 and 6; 5 and 9) were significantly similar to each other. Most of the samples were no similar or different than would be expected by chance. Likewise for the *Gammaproteobacteria* communities (Table 4.8).

Table 4.3: Raup-Crick Similarity Index Analysis of Bacterial Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.65	0.20	0.76	0.50	0.91	0.92	0.15	0.85	0.82	0.04	0.65	0.42	0.46	0.13	0.13	0.09	0.00
2			0.69	0.83	0.41	**0.99**	0.53	0.81	0.49	0.79	0.16	0.39	0.64	0.24	0.51	0.72	0.40	0.57
3				**0.97**	0.58	0.51	0.67	**0.99**	**0.95**	**0.95**	0.04	0.16	0.79	0.11	0.47	0.53	0.78	0.21
4					0.74	0.42	0.81	0.75	**1.00**	**1.00**	0.44	0.50	0.72	0.17	0.35	0.56	0.28	0.04
5						0.92	0.08	0.33	0.61	0.53	0.04	0.02	0.01	0.03	0.01	0.02	0.02	0.05
6							0.65	0.40	0.32	**0.97**	0.18	0.51	0.72	0.32	0.35	0.60	0.51	0.39
7								0.48	0.81	**1.00**	0.30	0.93	0.94	0.62	0.52	0.88	0.79	0.29
8									0.93	0.91	0.10	0.35	0.91	0.21	0.23	0.65	0.91	0.52
9										**0.98**	0.06	0.11	0.71	0.23	0.19	0.21	0.28	0.01
10											0.35	0.79	0.92	0.57	0.71	**0.95**	0.92	0.47
11												**0.99**	**0.95**	0.46	0.71	0.88	0.94	**0.99**
12													**1.00**	0.89	0.68	**0.95**	0.91	0.84
13														**0.99**	**0.95**	**1.00**	**1.00**	0.94
14															**1.00**	**1.00**	**0.99**	0.91
15																**1.00**	**0.99**	**0.99**
16																	**1.00**	**1.00**
17																		**1.00**
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

Table 4.4: Raup-Crick Similarity Index Analysis of *Acidobacteria* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.84	0.00	0.82	0.52	**0.98**	0.44	**0.98**	0.94	0.32	0.81	0.73	0.07	0.27	0.07	0.72	0.38	0.84
2			0.02	0.58	0.53	0.82	0.30	0.67	0.75	0.38	0.14	0.68	0.52	0.11	0.50	0.79	0.49	0.77
3				0.82	**0.95**	0.28	0.64	0.13	0.42	0.62	0.04	0.32	0.89	0.13	0.73	0.16	0.18	0.25
4					0.83	**1.00**	0.23	0.56	0.63	0.05	0.21	0.19	0.63	0.15	0.20	0.51	0.16	0.07
5						**0.99**	0.05	0.73	0.92	0.16	0.04	0.54	0.53	0.45	0.03	0.87	0.03	0.60
6							0.07	0.55	0.59	0.09	0.16	0.83	0.47	0.79	0.01	0.38	0.15	0.38
7								0.70	**0.97**	**0.99**	0.06	0.27	0.24	0.70	0.67	0.73	0.90	0.94
8									**0.97**	0.87	0.07	0.27	0.26	0.19	0.04	0.85	0.03	0.50
9										0.75	0.73	0.54	0.64	0.36	0.54	0.74	0.62	0.85
10											0.44	0.69	0.32	0.82	**0.98**	0.64	**0.96**	**0.98**
11												0.86	0.43	0.29	0.85	0.39	0.31	0.23
12													0.87	0.83	0.48	**0.99**	0.86	0.58
13														0.29	**0.96**	0.41	0.59	0.10
14															0.12	0.71	0.50	0.21
15																0.47	**0.99**	0.92
16																	0.63	0.68
17																		**0.96**
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

Table 4.5: Raup-Crick SimilarityIndex Analysis of *Actinobacteria* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.03	0.11	0.22	0.24	0.77	0.50	0.45	0.06	0.50	0.31	0.69	0.70	0.84	0.50	0.50	0.81	0.62
2			0.13	0.13	0.16	0.51	0.50	0.59	0.90	0.50	0.58	0.21	0.22	0.11	0.50	0.50	0.64	0.31
3				0.86	**0.98**	0.69	0.50	0.71	0.18	0.50	0.16	0.87	0.50	0.80	0.50	0.50	0.63	0.73
4					**0.95**	0.94	0.50	0.35	0.17	0.50	0.41	**0.99**	0.51	**0.95**	0.50	0.50	0.76	0.89
5						**0.98**	0.50	0.44	0.27	0.50	0.68	0.94	0.64	0.87	0.50	0.50	0.68	0.79
6							0.50	0.79	0.43	0.50	0.86	**0.96**	0.44	0.94	0.50	0.50	0.72	0.87
7								0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
8									0.22	0.50	0.25	0.58	0.21	0.43	0.50	0.50	0.65	0.77
9										0.50	0.22	0.50	0.50	0.20	0.50	0.50	0.38	0.26
10											0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
11												0.61	0.04	0.76	0.50	0.50	0.78	0.91
12													0.86	**1.00**	0.50	0.50	0.87	**0.97**
13														0.62	0.50	0.50	0.37	0.29
14															0.50	0.50	0.90	**0.99**
15																0.50	0.50	0.50
16																	0.50	0.50
17																		0.94
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

Table 4.6: Raup-Crick Similarity Index Analysis of *Alphaproteobacteria* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.50	**0.99**	0.05	0.52	0.82	0.21	0.56	0.85	0.50	0.17	0.28	0.35	0.11	0.13	0.50	0.09	0.03
2			0.94	0.25	0.70	**0.95**	0.39	0.82	0.92	0.50	0.41	0.23	0.60	0.22	0.33	0.50	0.29	0.75
3				0.57	0.78	**0.99**	0.35	0.67	0.84	0.50	0.19	0.43	0.89	0.43	0.83	0.50	0.23	0.48
4					0.60	0.57	0.66	0.10	0.27	0.50	0.13	0.41	0.36	0.07	0.90	0.50	0.76	**1.00**
5						0.80	0.77	0.68	0.90	0.50	0.56	0.80	0.27	0.01	0.57	0.50	0.50	0.55
6							0.85	0.31	**0.96**	0.50	0.01	0.06	0.13	0.19	0.62	0.50	0.23	0.49
7								0.43	0.28	0.50	0.18	0.29	0.38	0.28	0.31	0.50	0.47	0.34
8									0.12	0.50	0.77	**0.95**	0.43	0.02	0.58	0.50	0.85	0.07
9										0.50	0.10	0.21	0.60	0.21	0.64	0.50	0.15	0.63
10											0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
11												**0.95**	0.07	0.01	0.05	0.50	0.80	0.20
12													0.88	0.29	0.49	0.50	0.58	0.43
13														**1.00**	0.80	0.50	0.28	0.35
14															0.51	0.50	0.59	0.18
15																0.50	0.62	0.60
16																	0.50	0.50
17																		0.24
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

Table 4.7: Raup-Crick Similarity Index Analysis of *Betaproteobacteria* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.89	**0.98**	0.86	**0.97**	0.90	0.08	0.07	0.92	0.26	0.47	0.03	0.04	0.00	0.03	0.34	0.29	0.09
2			0.85	0.46	**0.98**	0.60	0.52	0.62	0.93	0.77	0.75	0.39	0.46	0.09	0.08	0.53	0.21	0.28
3				**0.99**	**0.98**	0.87	0.50	0.32	0.77	0.12	0.74	0.40	0.43	0.01	0.08	0.53	0.51	0.08
4					**0.99**	0.75	0.35	0.36	0.91	0.15	0.94	0.24	0.93	0.00	0.01	0.24	0.34	0.25
5						**0.99**	0.57	0.23	**1.00**	0.25	**0.99**	0.11	0.76	0.01	0.02	0.36	0.56	0.09
6							0.82	0.10	0.93	0.11	0.42	0.15	0.94	0.07	0.07	0.52	0.52	0.59
7								0.45	0.35	0.72	0.64	0.26	0.66	0.69	0.62	0.80	0.74	0.51
8									0.11	0.88	0.36	0.10	0.36	0.11	0.21	0.80	0.49	0.62
9										0.30	**0.95**	0.17	0.81	0.02	0.04	0.09	0.36	0.15
10											0.29	0.01	0.05	0.60	**0.97**	**0.99**	0.94	**0.97**
11												0.69	0.58	0.09	0.03	0.09	0.33	0.00
12													0.89	0.84	0.06	0.18	0.07	0.43
13														0.81	0.22	0.41	0.67	0.65
14															0.73	0.95	0.69	0.54
15																**0.99**	**1.00**	0.70
16																	**1.00**	**0.99**
17																		0.76
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

Table 4.8: Raup-Crick Similarity Index Analysis of *Gammaproteobacteria* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.50	0.20	0.55	0.63	0.23	0.50	0.20	0.11	0.18	0.13	0.26	0.02	0.52	0.02	0.50	0.00	0.18
2			0.91	0.32	0.58	0.63	0.50	0.39	0.20	0.38	0.22	0.30	0.04	0.39	0.07	0.50	0.13	0.78
3				**0.98**	**1.00**	**1.00**	0.50	0.36	0.48	0.38	0.48	0.62	0.05	0.38	0.09	0.50	0.45	0.76
4					**1.00**	**0.99**	0.50	0.29	0.18	0.20	0.17	0.73	0.04	0.22	0.03	0.50	0.04	0.22
5						**1.00**	0.50	0.57	0.38	0.31	0.13	0.75	0.13	0.34	0.06	0.50	0.09	0.25
6							0.50	0.64	0.75	0.50	0.42	0.65	0.12	0.51	0.14	0.50	0.29	0.34
7								0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
8									0.82	**0.97**	0.49	0.31	**0.96**	0.76	0.76	0.50	0.77	0.27
9										**0.98**	0.88	0.00	0.64	0.44	0.82	0.50	0.82	0.29
10											0.83	0.03	0.81	0.27	**0.97**	0.50	0.92	0.41
11												0.18	0.63	0.09	0.80	0.50	**0.98**	0.81
12													0.51	0.24	0.03	0.50	0.21	0.71
13														0.43	0.98	0.50	**0.98**	0.31
14															0.27	0.50	0.17	0.41
15																0.50	**0.99**	0.42
16																	0.50	0.50
17																		0.86
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

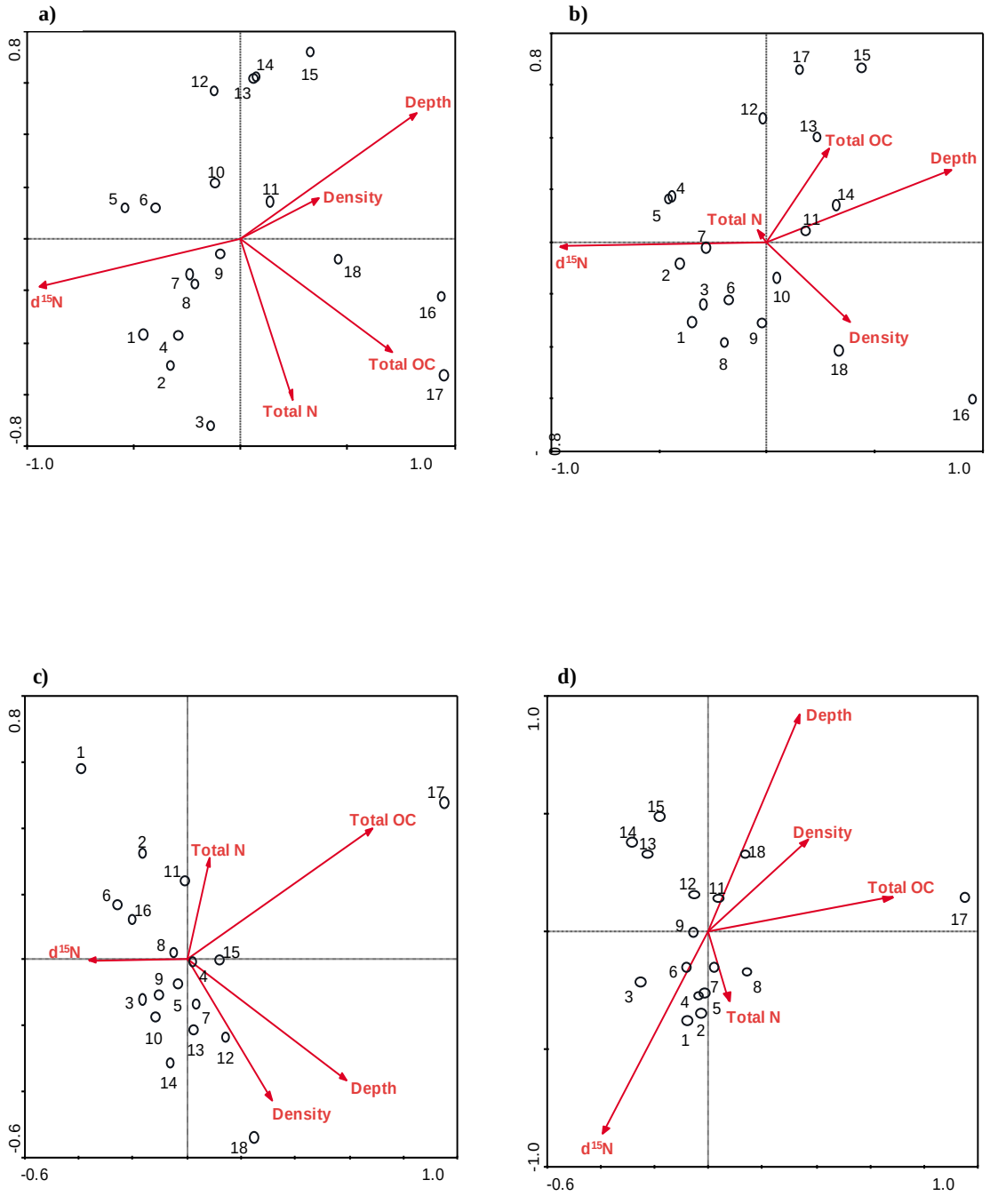
Table 4.9: Raup-Crick Similarity Index Analysis of *Archaea* Communities

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1		0.47	0.19	0.07	0.34	0.43	0.83	0.86	0.72	0.13	0.04	0.28	0.17	0.19	0.47	0.46	0.06	0.47
2			0.27	0.11	0.27	0.39	0.54	0.45	0.85	0.08	0.03	0.01	0.03	0.04	0.04	0.14	0.15	0.16
3				0.36	0.39	0.61	0.05	0.42	0.31	0.43	0.17	0.28	0.33	0.57	0.55	0.34	0.12	0.36
4					**0.96**	0.49	0.68	0.36	0.64	0.28	0.49	0.11	0.54	0.06	0.65	0.25	0.22	0.54
5						0.82	0.73	0.53	0.84	0.47	0.27	0.53	0.25	0.32	**0.99**	0.55	0.30	0.58
6							0.26	0.74	0.50	0.58	0.17	0.58	0.06	0.11	0.77	0.31	0.18	0.06
7								**0.97**	**0.97**	0.68	0.53	0.16	0.72	0.44	0.63	0.94	0.86	0.93
8									0.93	0.20	0.17	0.38	0.32	0.25	0.61	0.32	0.27	0.32
9										0.01	0.00	0.07	0.15	0.58	0.04	0.15	0.06	0.14
10											**1.00**	0.59	**0.99**	0.88	**1.00**	**1.00**	**1.00**	**0.99**
11												0.80	0.90	0.49	**0.99**	**0.98**	**0.98**	**1.00**
12													0.62	0.76	0.72	0.58	0.82	0.59
13														**0.99**	0.92	**1.00**	**1.00**	**1.00**
14															0.57	**0.96**	0.87	0.83
15																**0.99**	0.93	**0.99**
16																	**1.00**	**1.00**
17																		**1.00**
18																		

The underlined value = significant dissimilarity ($S_{RC} \leq 0.05$); the highlighted value in red = significant similarity ($S_{RC} \geq 0.95$); the rest of the values = similarity occurred by chance ($0.05 < S_{RC} < 0.95$)

4.3.4. Multivariate analysis of microbial communities' structure

Statistical analysis of RDA/CCA with Monte Carlo permutation test was employed to evaluate the relationship between microbial community structures (the evenness of species distribution) and environmental variables. In an ordination diagram of RDA/CCA, circles with numbers represent the community profile in that particular depth of the sediment core while arrows represent environmental variables that determines a direction or 'axis'. The length of each arrow also indicates the importance of one environmental variable to the community (ter Braak, 1986). As revealed by the multivariate analysis, sediment depth appeared to have significant effect on most of the taxa community structures which showed $p < 0.05$, including bacteria, *Alphaproteobacteria* and *Betaproteobacteria* as well as the archaeal community structure (Figure 4.3a, 4.3c, 4.3d, 4.3e, 4.3g; Table 4.10). For *Acidobacteria* (Figure 4.3b; Table 4.10) and *Gammaproteobacteria* (Figure 4.3f; Table 4.10), their community structures showed no positive relationship with depth and instead, they were significantly affected by $\delta^{15}N$ in Suigetsu sediment ($p = 0.002$). For *Betaproteobacteria*, sediment density and depth were significant ($p = 0.06$; 0.02 respectively) in shaping the community structure. For the archaeal community, apart from sediment depth, total organic carbon ($p = 0.082$) and total nitrogen ($p = 0.08$) in Suigetsu sediment appeared to be drivers in changing its community structure.


a)
Depth
Density
Total OC
Total N
d15N
b)
Total OC
Depth
Total N
Density
d15N
c)
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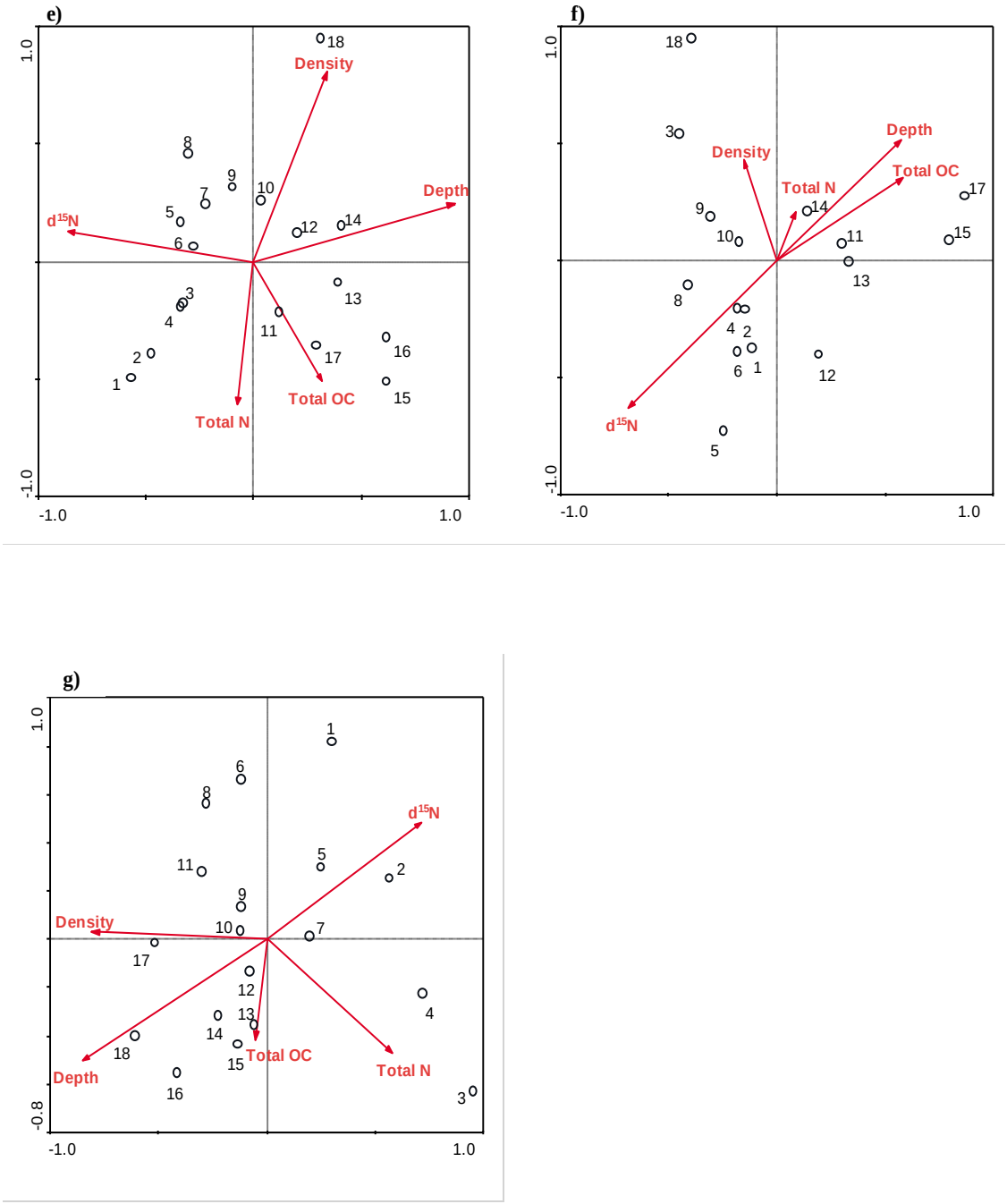


Figure 4.3: Multivariate analysis of community profiles. a) RDA analysis of bacteria, b) CCA analysis of *Acidobacteria*, c) RDA analysis of *Actinobacteria*, d) CCA analysis of *Alphaproteobacteria*, e) CCA analysis of *Betaproteobacteria*, f) CCA analysis of *Gammaproteobacteria*, g) CCA analysis of archaeal. Open circles with numbers represent sediment samples from core A (N) 01 to A (N) 46 in sequential orders. Arrows indicate environmental variables in which Depth: sediment depth, Total OC: Total organic carbon, Total N: Total nitrogen, $d^{15}N$: ratio of stable isotope of nitrogen $\delta^{15}N$, and Density: sediment density. 1= A (N) 01; 2= A (N) 03; 3= B (N) 05; 4=B (N) 07; 5= B (N) 11; 6= A (S) 13; 7= A (N) 16; 8= B (N) 17; 9= B (N) 19; 10= B (N) 21; 11= A (N) 24; 12= A (N) 28; 13= A (S) 30; 14= A (S) 32; 15= A (S) 35; 16= A (N) 39; 17= A (N) 43; 18= A (N) 46

Table 4.10: Evaluation of environmental factors against prokaryotic community structure based on CCA analysis

	Conditional Effects						
	Bac.	***Acido.***	***Actino.***	***α-pro.***	***β-pro.***	***γ-pro.***	***Archaea***
Variable	*p*	*p*	*p*	*p*	*p*	*p*	*p*
Depth	0.662	0.240	0.496	**0.006**	**0.002**	0.492	**0.004**
TOC	0.354	0.512	**0.096**	0.328	0.834	0.354	**0.082**
$\delta^{15}N$	**0.002**	**0.002**	0.280	0.452	0.424	**0.002**	0.536
Density	0.854	0.110	0.878	0.142	**0.060**	0.372	0.206
TN	0.790	0.638	0.216	0.488	0.856	0.168	**0.080**
Water co.							

Highlighted value of $p < 0.05$ in red represents significant correlation between microbial community structure and environmental variables while $p > 0.05 < 0.1$ indicates weak correlation between environmental factors and microbial community structure. Depth: sediment depth, TOC: Total organic carbon, $\delta^{15}N$: ratio of stable isotope of nitrogen, Density: sediment density and TN: Total nitrogen. Data for water content was excluded as the variation inflation factor (VIF) is higher than 20.

4.3.5. Pearson correlation coefficient analysis of microbial diversities

Pearson correlation coefficient analysis was also conducted to assess the linear relationship between the prokaryotic diversity and environmental variables (Table 4.11). The bacterial diversity, overall, was significantly affected by depth (p = 0.008), $\delta^{15}N$ (p = 0.008) and weakly affected by water content (p = 0.061). Several environmental factors have also shown to affect the diversity of *Actinobacteria* and *Gammaproteobacteria* simultaneously which include sediment depth (p = 0.001, 0.009) and nitrate levels (p = 0.002, 0.005). Total nitrogen has also exerted a significant impact in shifting the *Actinobacteria* diversity (p = 0.015) while *Gammaproteobacteria* diversity was significantly affected by sediment's water content (p = 0.035). In addition to this, sediment density also exerted a weak force in altering their diversity, as indicated by p = 0.091 and p = 0.092, respectively. The diversity of *Acidobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* as a whole was not shaped by any of the environmental variables from Suigetsu sediment, except for water content that has exerted a weak force on the diversity of *Alphaproteobacteria* community (p = 0.079). For archaeal community, its diversity was significantly influenced by sediment density (p = 0.013) and water content (p = 0.006) while sediment depth has a minor effect in shaping its diversity (p = 0.084).

Table 4.11: Significant evaluation of environmental factors against prokaryotic diversity based on Pearson Correlation

Env. Variables		***Bac.***	***Acido.***	***Actino.***	***α-pro.***	***β-pro.***	***γ-pro.***	***Arch.***
Depth	**Coef.**	-0.603	-0.161	-0.715	-0.286	0.023	-0.597	-0.418
	p	**0.008**	0.524	**0.001**	0.250	0.926	**0.009**	**0.084**
TOC	**Coef.**	0.414	-0.115	-0.301	-0.276	-0.102	-0.349	-0.023
	p	**0.087**	0.651	0.225	0.267	0.688	0.156	0.927
$\delta^{15}N$	**Coef.**	0.605	0.369	0.673	0.370	0.177	0.633	0.322
	p	**0.008**	0.132	**0.002**	0.130	0.433	**0.005**	0.193
Density	**Coef.**	-0.268	-0.094	-0.410	-0.272	0.075	-0.409	-0.570
	p	0.283	0.711	**0.091**	0.275	0.768	**0.092**	**0.013**
Water	**Coef.**	0.450	0.145	0.131	0.425	-0.283	0.499	0.620
	p	**0.061**	0.565	0.604	**0.079**	0.255	**0.035**	**0.006**
TN	**Coef.**	-0.151	-0.233	0.564	-0.130	0.002	-0.117	0.227
	p	0.550	0.352	**0.015**	0.608	0.994	0.643	0.365

Highlighted *p* values in red = significant correlation (< 0.05) while underlined *p* value in black = weak correlation ($p > 0.05 < 0.1$). A positive coefficient value = positive relationship while negative coef. value = negative correlation. Depth: sediment depth, TOC: Total organic carbon, $\delta^{15}N$: ratio of stable isotope of nitrogen, Density: sediment density, Water co: water content and TN: Total nitrogen. Coef. = Pearson Correlation coefficients and *p* = p value.

As a whole, these statistical analyses had shown that different environmental factors may exert different forces in altering the community structure and the diversity of prokaryotic communities in Lake Suigetsu sediment. In fact, prokaryotic community structure and its diversity could be substantially affected by one or more parameters simultaneously which could make the analysis more complicated and not as straightforward.

4.4. Discussion:

Soils have distinctive biological, physical and geochemical properties in comparison to other habitats and they are environments which harbour the most diverse microbial communities, including novel bacterial species (Torsvik and Øvreås, 2002). In the anoxic and stratified environment of meromictic lakes, diverse bacteria have been found in both the water columns (Cytryn *et al*., 2000, Humayoun *et al*., 2003; Lehours *et al*., 2005; 2007) and the sediments (Bhattarai *et al*., 2012; Borrel *et al*., 2012; Ravasi *et al*., 2012; Klepac-ceraj *et al*., 2012). However, microbial communities in the sediment which have close association with particles are known to be fundamentally different from the free-living microbial populations in the water column (Swan *et al*., 2010). These differences are due to the heterogeneous nature of sediment communities, in terms of depth, age, nutrient availability and sources of reducing power compared to those formed in the pelagic communities (Borrel *et al*., 2012; Klepac-ceraj *et al*., 2012; Koizumi *et al*., 2004; Swan *et al*., 2010). The environmental gradients in the sediments therefore will have higher impact in shaping microbial communities and affecting their abundances.

In this study, we hypothesised that bacterial fossil DNA can be used as the proxy in conjunction with molecular techniques to explore possible paleoenvironmental indicators for past climate change in the freshwater sedimentary records of meromictic Lake Suigetsu. Fossil DNA has been demonstrated to overcome the limitations associated with traditional standard biomarkers (i.e. fossilizing protists, diatoms, lipids and pigments) such as a lack of diagnostic features (Coolen *et al*., 2004; Coolen *et al*., 2008; Coolen and Gibson, 2009). A study by Novitsky (1987) disclosed that microbial communities in deep sediments are mainly non-growing cells that are in a dormant state. Fully hydrated DNA can be degraded to short fragments spontaneously through oxidation and hydrolysis after few thousands of years (Coolen and Overmann, 1998, 2007; Hofreiter *et al*., 2001; Lindahl, 1993; Poinar *et al*., 1996; Richter, Park and Amest, 1998). However under ideal conditions, DNA can persist for tens or hundreds of thousands of years (Coolen and Gibson, 2009; Coolen and Overmann, 1998; 2007).

Meromictic lakes sediments such as those of Lake Suigetsu have been found to provide conditions for excellent preservation of fossil DNA (Coolen *et al*., 2004; Coolen and Gibson, 2009). The sediment is anaerobic, highly concentrated with

inorganic materials and with undisturbed laminated sediments (Coolen and Gibson, 2009). Besides meromictic settings, several other factors can also contribute to DNA persistence in Suigetsu sediments. Cold environments are definitely one of the factors that have been reported to better preserve DNA (Coolen and Overmann, 2007; Hofreiter *et al*., 2001; Lindahl, 1993; Willerslev and Cooper, 2005). In addition, Suigetsu sediments are composed of massive inorganic clay layers and intermittently finely laminated organic clay laminae (Nakagawa *et al*., 2012). These minerals have adsorptive capabilities for organic and inorganic materials via covalent or electrostatic bonding (Davidson and Janssens, 2006; Lorenz and Wackernagel, 1994) that can chemically protect them from degradation, and thus they can prolong the half-life of DNA (Lorenz and Wackernagel, 1994; Romanowski *et al*., 1991). DNA can be adsorbed onto several minerals such as clay, quartz, feldspar, heavy minerals and even humic substances to form stable complexes (Crecchio and Stotzky, 1998; Demanèche *et al*., 2001; Khanna and Stotzky, 1992; Lorenz and Wackernagel, 1994; Koizumi *et al*., 2003; Romanowski *et al*., 1991). Clays have the highest net of negative charge and a DNA binding capability of 700-fold higher than that of quartz sand (Lorenz and Wackernagel, 1987, 1994). Besides, cation concentrations and pH play important roles in soils and sediments as the determining factors for strong DNA adsorption. The repulsion forces can be weakened by high cation concentrations (i.e. Na^+, K^+, NH_4^+, Mg^{2+} or Ca^{2+}). Consequently, in environments with high level of NaCl concentrations, more DNA will be saturated onto clays' surfaces (Lorenz and Wackernagel, 1987, 1994) and nucleases that are capable of cleaving the nucleic acids phosphodiester bonds will also be inhibited (Hofreiter *et al*., 2001; Lindahl, 1993; Lorenz and Wackernagel, 1994). Therefore high NaCl concentration can extend the preservation of fossil DNA in sediments. Likewise, in low pH environment (pH < 5), DNA binding can become improved by up to 200 µg per gram of soil as protonation of bases takes place which causes DNA to become positively charged (Crecchio and Stotzky, 1998; Khanna and Stotzky, 1992; Lorenz and Wackernagel, 1994; Romanowski *et al*., 1991).

Most of the recovered fossil DNA fragments are relatively short which is between 1000 to 500 base pairs (bp). Many studies have shown the successful application of such fragments in the study of microbial communities of similar environments (Gorlenko *et al*., 2010; Gugliandolo *et al*., 2011; Koizumi *et al*., 2004; Willerslev and Cooper 2005). Even so, all PCR based techniques are frequently associated with potential biases (Lueders and Friedrich, 2003; Reysenbach *et al*., 1992;

Marzorati *et al*., 2008). First, the choice of DNA extraction method can be the key limitation because the yield of nucleic acids is usually allied to the efficiency of cell lysis and the elimination of inhibitors (Carrigg *et al*., 2007). Humic acids are one of the main inhibitors largely found in environmental samples. They are the major organic constituents commonly found in soil (Wheeler and Stahl, 1996) and thereby could hinder PCR amplification. Moreover, the use of group specific primer sets to amplify extracted environmental DNAs which contain multi-template cannot guarantee a sequence common in the targets if the primer has one mismatch with some targets and hence would generate an even greater bias with very low amplification efficiency (Kanagawa, 2003).

In addition, a greater bias in PCR amplification is the differential primer affinity or priming given that this is largely due to the dissimilarities between primary and secondary DNA structure at the potential target sites. These differences can potentially lead to the occurrence of selectivity and preferential amplification of certain members of a gene family (Head *et al*., 1998; Muyzer and Smalla, 1998). Preferential amplification can also occur when PCR amplification involves two successive PCR reactions (Suzuki and Giovannoni, 1996). Ben-Dov *et al*. (2006) indicated that high rigidity primer annealing can sometimes result in selective amplification of some sequences even though there are only small differences in the sequence of universally conserved regions. Thus, the claimed universality of the commonly used primers is not absolute.

In this study, short fossil DNA sequences are fairly useful for molecular techniques like PCR-DGGE that required only 500 bp of PCR amplicons for analysis (Muyzer and Smalla, 1998; Myers *et al*., 1985). The genomic DNA fingerprinting of DGGE allows the estimation of the number and abundance of predominant taxa in the sample based on DGGE band number, band intensity and position. Despite the complex DGGE banding patterns that reflect high microbial diversity from sediments which are generated when bacterial primers are used, only the major populations are represented in DGGE patterns. The less abundant yet potentially important bacterial species may not be detected by this molecular technique (Heuer *et al*., 1997). The use of the nested PCR approach with specific primers, as in this study, has been suggested to increase sensitivity, which makes it possible to visualize those species that are present in lower numbers (Boon *et al*., 2002; Liu *et al*., 2007). Specific primers are useful for direct

amplification and analysis of the 16S rDNA genes of defined groups within the complex microbial community in Lake Suigetsu sediment. The analysis of group-specific PCR-DGGE was suggested to be a valuable tool that provides rapid comparison data that can monitor the structure and dynamics of microbial populations over time or under the influence of environmental changes (Boon *et al*., 2002). Nested PCR comprises of two rounds of PCR amplification. In the first round of PCR, specific fragments were amplified using group specific primers while in the second round, a set of bacterial primers with a GC-clamp were utilised which is essential for DGGE analysis to reduce and standardise the length of the specific fragments. The use of a nested approach can extend the possibilities of DGGE in microbial community analysis as it can better reveal small changes within or differences between microbial communities. This approach has been utilised to investigate specific microbial groups including *Alpha*- and *Betaproteobacteria* (Henckel *et al*., 1999), *Actinomycetes* (Heuer *et al*., 1997), *Gammaproteobacteria* (Gomes *et al*., 2001), ammonia-oxidising bacteria (Kowalchuk *et al*., 1998), *Archaea* (Øvreås *et al*., 1997) and fungi (Smit *et al*., 1999).

However, in our study, several taxa such as *Deltaproteobacteria*, *Bacteriodetes* and *Cyanobacteria* that have been detected previously in the water column of Lake Suigetsu (Kondo *et al*., 2006; Kondo *et al*., 2009; Okada *et al*., 2007) were unable to be detected despite numerous modifications of protocols. Similarly, the recent study by Mori *et al*. (2010) also found no SRB (related to *dsrA* genes) that belong to the phylum *Deltaproteobacteria* in the lake waters and this could be that the amount of DNA extracted from these particular phyla was below the detection limit. Besides, the specificity of group-specific primers is largely dependent on the availability of known sequences from cultured isolates in the database, therefore, may not be adequate to reflect the entire pool of 16S rDNA sequences in the environment particularly for the analysis of natural microbial communities (Heuer *et al*., 1997; Purkhold *et al*., 2000). Thus, in this study, all the amplified bands in the DGGE gels may not entirely represent the true members of its respective group and further approach such as sequencing of random DGGE bands for each primer set is necessary to confirm whether the sequences corresponded to bacteria belonging to the respective amplified group.

In this study, PCR-DGGE has successfully revealed the presence of bacteria, phyla *Proteobacteria* (subclass alpha, beta and gamma), *Acidobacteria*, *Actinobacteria* and *Archaea* communities. The assessment of the bacterial community is necessary as it

provides an overview of overall microbial community patterns in the deep sediment of Lake Suigetsu. Although some of these taxa were absent from certain sediment depths, as a whole, PCR-DGGE showed that they are distributed throughout the entire depth of the 73 m sediment core with an uneven dispersal pattern. These populations are even detectable in the very deepest sediment samples of the core from 140,000 BP years. A study by Biderre-Petit *et al*. (2011) indicated that microbial activities in lake sediments are much greater compared to the lake waters, therefore the observation of high microbial diversity in Lake Suigetsu sediment core should be expected. By comparing with other meromictic lake sediments in Japan, Koizumi *et al*. (2004) showed that there was high microbial diversity in the sediment of Lake Kaiike however their distributions' pattern was consistent when sediment depths increased, which is in contrast to our findings. The differences were that they explored and compared the microbial community at short sediment intervals of 0.3 to 2 cm whilst this study compared sediment samples at a rather distant depth altogether (4 m intervals). The study by Zhou *et al*. (2004) showed that the vertical distribution of microbial species that were a few meters apart was mostly different (> 90%). Notably, the microbial community in Kaiike sediments were compared between young sediments while in this study, the comparison of microbial community was between young and older sediments. Hence, resulting in greater differences in microbial distribution patterns when young and older sediments were compared. Such observations could also imply that microbial community pattern do not change variably but gradually over a long period of time.

In addition, the diversity of the bacterial community in Suigetsu sediment was detected higher in the top cores than in the deepest. According to Novitsky (1987), between the interface and the sediment below, there is a sharp boundary and usually the biomass and its production are quite homogeneous on the top 5 cm sediment. He found large numbers of active microbial community at the interface, with specific growth rate and productivity greater than that measured in the deeper-sediment horizons, likewise near the coastal subtropical sediment (Novitsky and Karl, 1986). It has been suggested that large amount of cellular biosynthesis, heterotrophic activity and organic matter mineralisation could occur at the top few millimetre of sediment interface as the community at this region obtain more nutrient input than the communities in deeper sediment, given that the carbon and energy input to this sediment system is via the water column (Novitsky, 1987).

Acidobacteria is one of the most widely distributed bacterial groups in the sediment core. Members of this phylum can be found in a wide variety of environments such as soils (Janssen, 2006; Kielak *et al*., 2009; Lee *et al*., 2008), sediments (Dong *et al*., 2006; Jiang *et al*., 2006; Tamaki *et al*., 2005), freshwater (Barns *et al*., 1999), lakes (Kleinsteuber *et al*., 2008, Klepac-ceraj *et al*., 2012), marine (Barns *et al*., 1999), wastewater systems (Boon *et al*., 2002), acidic mines (Baker and Banfield, 2003; Lear *et al*., 2009), peats (Dedysh *et al*., 2006) as well as polluted environments (Nogales *et al*., 2001; de Cárcer *et al*., 2007). In soils and sediments, *Acidobacteria* can contribute up to 50% of the community as shown in clone libraries (Branco *et al*., 2005; Barns *et al*., 2007; Dedysh *et al*., 2006; Janssen 2006; Kielak *et al*., 2009; Penn *et al*., 2006; Sievert *et al*., 2000; Ward *et al*., 2009). A study based on quantitative PCR found that the relative abundance of *Acidobacteria* was negatively correlated with soil carbon availability (Fierer *et al*., 2007) and previous studies of microbial succession in soil have showed that *Acidobacteria* are more abundant in older soils than in young soils (Nemergut *et al*., 2007; Tarlera *et al*., 2008). Besides, Newton *et al*. (2011) also indicated that *Acidobacteria* are significant in freshwater sediments. Therefore, these findings may support their distribution in Lake Suigetsu sediment as Lake Suigetsu is high in inorganic materials, lacks oxygen and nutrients, low temperature and mild to moderate acidic environments (Matsuyama and Sajio, 1971) which are the conditions that favour the growth of *Acidobacteria* (Eichorst *et al*., 2007; Kleinsteuber *et al*., 2008; Klepac-ceraj *et al*., 2012; Philippot *et al*., 2010).

Proteobacteria is the second largest bacterial group that is detected in Suigetsu sediment core. Members of this phylum are also widely detected in various environments such as sediment (Briée *et al*., 2007; Burkert *et al*., 2003), freshwater (Glöckner *et al*., 2000; Newton *et al*., 2011; Tamaki *et al*., 2005), marine (Sogin *et al*., 2006), meromictic lakes (Klepac-ceraj *et al*., 2012; Meyer *et al*., 2011), alkaline soda lake (Wani *et al*., 2006) as well as Antarctic lake (Pearce *et al*., 2003). The findings here show that the beta subclass is the most diverse followed by alpha and gamma subclasses (Table 4.2). The lower abundance of *Alphaproteobacteria* population in the sediments of Lake Suigetsu may be due to high grazing pressure exerted from protozoans that preferentially graze on this taxon (Mori *et al*., 2013).

Alphaproteobacteria have been found to preferentially grow in more acidic environments between pH 5.5 to 6.0 (Tamaki *et al*., 2005) whereas *Betaproteobacteria*

favour a more alkaline condition than acidic environments (Lindström, *et al*., 2005; Šimek *et al*., 2010). Additionally, *Betaproteobacteria* abundance has a positive correlation to the low molecular weight alga-derived substrates released near the upper waters (Newton *et al*., 2011). This can be due to the complicated physiological properties of *Betaproteobacteria* that required a number of essential electron donors and acceptors to sustain their growth (Kojima and Fukui, 2011). Therefore, the population of the beta subclass may decrease with increasing water depths and eventually may result in lower deposition of populations at the bottom sediment of Lake Suigetsu over time. Some *Betaproteobacteria* strains are found in high levels in the hypolimnion anoxic region (Salcher *et al*., 2008). This suggests that those *Betaproteobacteria* detected in the deeper sediments of Lake Suigetsu may have been indigenous to the anoxic zone at the bottom lake waters before deposition and they are likely to be the facultative anaerobes from a freshwater lake (Hahn *et al*., 2010).

In the Suigetsu sediment core, *Gammaproteobacteria* had lower diversity in comparison with *Alpha-* and *Betaproteobacteria*. The community patterns were relatively similar at each sediment depth and this taxon was undetectable in the deeper sediment samples. *Gammaproteobacteria* populations might be the transient members of lake communities brought in from surrounding environment (Newton *et al*., 2011). In this case, it is possible as Lake Suigetsu is adjacent to Lake Kugushi in which Lake Kugushi is a shallow haline lake and small amount of seawater have been reported to flow into Lake Suigetsu (Matsuyama and Sajio 1971), therefore *Gammaproteobacteria* may be introduced into Lake Suigetsu via seawater intrusion. The small populations of *Gammaproteobacteria* could also be the common lake members that already exist in Lake Suigetsu at low abundance, hence can also lead to the consistency of community patterns on DGGE gels. Furthermore, *Gammaproteobacteria* are copiotrophs largely found in nutrients and carbon rich environments (Zavarzin *et al*., 1991). Thereby, the conditions in Lake Suigetsu sediments may not favour their proliferation.

Actinobacteria are commonly recovered from various freshwaters habitats and some from marine environments (Glöckner *et al*., 2000; Jensen *et al*., 1991; Maldonado *et al*., 2005; Newton *et al*., 2011; Warnecke *et al*., 2004). Their abundance has found to be quite consistent across various lake types worldwide. Among the phyla discovered, *Actinobacteria* was the least diverse and abundance in the Suigetsu sediment core although some of the species were still detectable in low abundance in the deepest

sediment depths. The acI lineage within the phylum is one of the most abundant and exclusive lineages in freshwater (Newton *et al*., 2007). Their wide distribution was postulated to have correlation to their small cell size and S layer cell wall compositions that can protect them from protozoans' predation (Newton *et al*., 2007; Newton *et al*., 2011; Warnecke *et al*., 2004). Further work is required to determine whether the two main strains that appear at the bottom of DGGE gels that are detected in almost every sediment depth are from this acI lineage. In addition, some *Actinobacteria* are able to form spores that allow them to withstand mild heat, UV radiation and desiccation under a long period of time (Goodfellow and Williams 1983; Newton *et al*., 2011; Ventura *et al*., 2007). Such capability may also contribute to why *Actinobacteria* are detectable even in the paleo-sediments of Lake Suigetsu.

Moreover, Goodfellow and Williams (1983) pointed out that most of the *Actinobacteria* species are aerobic and level of oxygen concentration often has linear relationship with increasing sediment depth. Some studies have shown that *Actinobacteria* are abundant at the oxygenated surface waters and decrease with oxygen concentration level in the anoxic bottom waters (Glöckner *et al*., 2000; Newton *et al*., 2011). Humayoun *et al*. (2003) also identified compatible observations where dominant sequences of *Actinobacteria* from oxygenated surface water decreased in the chemocline and were eliminated in the anoxic monimolimnion bottom region of the meromictic Mono Lake. This suggests that oxygen concentration is a fundamental element to *Actinobacteria* community thereby leading to a low actinobacterial deposition and persistence in the deeper sediment of Lake Suigetsu.

In Lake Suigetsu sediment, archaeal populations are detectable by PCR-DGGE as well. *Archaea* are frequently reported to be ubiquitous and abundant in marine habitats (DeLong 2003; Francis *et al*., 2005; Galand *et al*., 2009; Urakawa *et al*., 1999) as well as in soils (Leininger *et al*., 2006), hot springs (Keller and Zengler, 2004), freshwater habitats and meromictic lake sediments (Bhattarai *et al*., 2012; Koizumi *et al*., 2004; Nam *et al*., 2008). In deep sediment samples of Lake Suigetsu, archaeal populations were detected. They are scattered unevenly in every sediment depth examined but with a lower diversity compared to bacterial population. Studies have shown that *Archaea* in general are quite predominant in saline lakes sediments (i.e. Lake Chaka, Lake Qinghai, Solar Lake and Maras Salterns) but not in freshwater's, except for *Crenarchaeota* which have shown to be quite exclusive in freshwater habitats

(Cytryn *et al*., 2000; Maturrano *et al*., 2006; Jiang *et al*., 2007; Jiang *et al*., 2008). They are noted as a minority group in the total prokaryotic community in freshwater sediments (Borrel *et al.*, 2012). Valentine (2007) postulated that bacterial diversity, adaptability and prevalence are greater than the *Archaea* in freshwater because of the minor energy stress that is less persistent in the freshwater compared to marine.

In Suigetsu sediment core, archaeal diversity in the upper sediment depths of the WAMX biome from A (N) 01 to B (N) 07 were almost twice as diverse than the communities found in the deeper sediment depths in the 'unknown' biome zone from B (N) 21 to A (N) 46 (Table 4.2). This is in agreement with the study by Nam *et al*. (2008) who reported that archaeal diversity is greater on the upper sediments of Lake Hovsgol than in the deeper sediments. In contrast, Koizumi *et al*. (2004) showed that archaeal communities largely increased with sediment depth in saline Lake Kaiike. This could be that saline environments favour the growth of *Archaea* more than in freshwater lake. Borrel *et al*. (2012) noticed that there were no significant vertical variations in the abundance of the overall *Archaea* lineages in deep freshwater sediment. The only depth-related changes that occurred were in the composition and structure of the archaeal community but with no clear evidence of how each archaeal lineage is affected by depth in the sediment. In addition to this, they also found that the archaeal communities at the surface sediment are mostly methanogens which are involved in methanogenesis whereas in the deeper sediment the archaeal community consisted of mainly uncultivated lineages. However, this observation is different from the study carried out by Nam *et al*. (2008) where they detected no methanogens in the deeper lake sediment and speculated that methanogenesis may not occur in the sediments when sulfate reduction takes place. Lovley and Klug (1983) indicated that SRB will outcompete methanogens for reducing power, therefore the presence of methanogen in Lake Suigetsu is highly unlikely given that SRB has been largely reported to be present in the lake water (Kondo *et al*., 2000; 2006; 2009) as well as the sediment surface of Lake Suigetsu (Kondo and Butani, 2007). The archaeal communities detected in Suigetsu sediments may either be the acidophiles, nitrifiers (Valentine, 2007) or uncultivated lineages.

Following the discussion above, microbial diversity and community structure are evidently not controlled simply by a single parameter but numerous environmental factors that include sediment age/depth and nutrient availability. Microorganisms are

responsive to their surrounding environments and there is a certain level of impact on the microbial community from the physical and chemical properties of the environments (Hossain and Sugiyama 2011). Statistical analyses such as CCA and Pearson correlation are, therefore, employed to cross-examine the relationship between the bacterial community structure and environmental parameters. Sediment samples that were analysed are matched to the recorded pollen data by Nakagawa *et al*. (2002, 2005). Pollen data essentially captures the information on the biome that contains the palaeoclimatic information at local and large regional scales and this biome record can be used as a reference in searching for potential paleoenvironment biomarkers (Gotanda *et al*., 2002; Nakagawa *et al*., 2005; Zhou *et al*., 2005). Pollen grains are generally produced in large quantities and they are well-preserved in lakes sediments due to their extremely resistant walls that can resist decay (Blackmore 2007). Furthermore, during seasonal changes, vegetation shifted accordingly and different pollen grains are produced (Nakagawa *et al*., 2002). Therefore, pollens can be calibrated directly against climatic parameters on account of their quantitative nature (Webb and Bryson, 1872). In various parts of the world, pollen records have been widely used in establishing vegetation (biomes) and reconstructing past climate (Bartlein *et al*., 1984; Peyron *et al*., 1998, 2000; Tarasov *et al*., 1999; Zhou *et al*., 2005), including Japan (Gotanda *et al*., 2002; Takahara *et al*., 2000) and Greenland (Fredskild and Wagner, 1974).

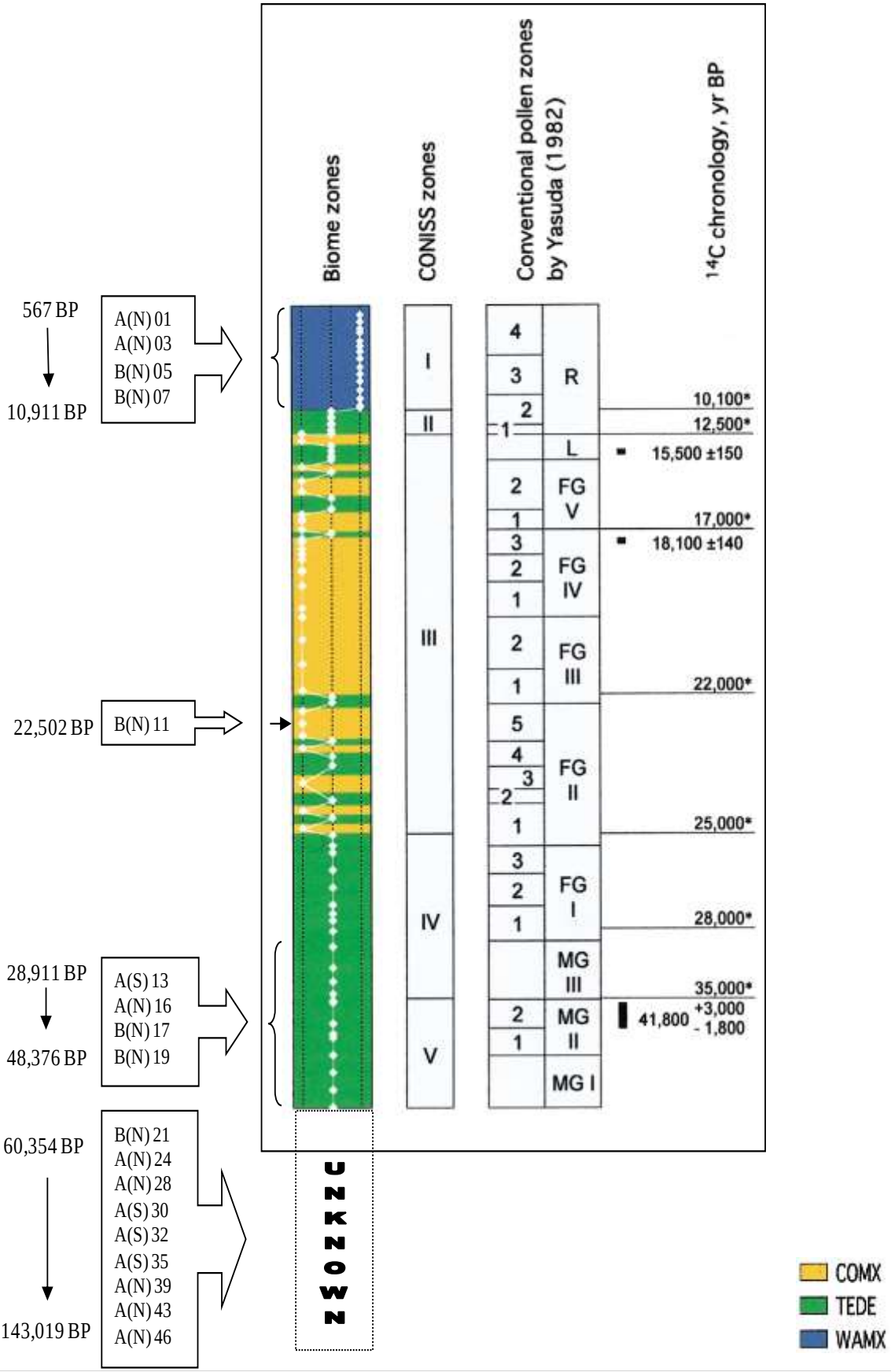


Figure 4.4: These are the biomes reconstructed based on the pollen analysis from Lake Mikata and this diagram was extracted from the paper of Gotanda *et al*. (2002). 18 core samples were matched accordingly to the biome zones based on age. WAMX = warm mixed forest, TEDE = temperate deciduous forest and COMX = cool mixed forest. Biome zones before 48,500 yr BP are yet to be resolved.

Our collected sediment samples are also categorized into different biome zones according to the reconstructed biome based on Lake Mikata's pollen records as shown in Figure 4.4. The reconstructed biome of Lake Mikata is used as it has a much broader chronological coverage that encompasses the last *ca*. 47,000 years (Gotanda *et al*., 2002). Besides, this existing biome record of Lake Mikata can also be used as the representation of Lake Suigetsu biome as both lakes are adjacent to one another and thereby should share the same vegetation. The upper cores from A (N) 01 to B (N) 07 represent samples from the Holocene period which falls in the biome of warm mixed forest (WAMX) that is between 567 yrs BP to 10,911 yrs BP. The following biome represented by core B (N) 11 was in the reconstructed zone between temperate deciduous forest (TEDE) and cool mixed forest (COMX) which was for year 22,502 BP whereas for core A (S) 13 to B (N) 19, between 28,911 yrs BP to 48,376 yrs BP, the biome identified was TEDE. The biome for cores B (N) 21 to A (N) 46, from 60,354 yrs BP to 143,019 yrs BP however, is difficult to identify as the biomes based on Lake Mikata for these periods are yet to be resolved.

Similarly, for Lake Suigetsu, the vegetation that occurred from 50,000 yrs BP to 143,000 yrs BP in the deeper cores have not been identified as most of the studies mainly focus on the climate reconstruction of the Last Quaternary (Gotanda *et al*., 2002, Nakagawa *et al*., 2005; Kossler *et al*., 2011). Nakagawa *et al*. (2012) pointed out that the composite depth between 4601.4 cm to 6375.2 cm is not laminated as during this period, the lake was still shallow and not deep enough to form an abiotic bottom water environment (Takemura *et al*., 1994). In addition, the composite depth below 6375.2 cm down to the core bottom are composed of recurrences of peat, massive inorganic clay layers, and intermittently laminated organic clays which is inferred as the alternating fluvial and shallow water environments that occurred after the initial tectonic formation of the basin (Nakagawa *et al*., 2012).

Multivariate analyses of CCA and RDA revealed that the community structure for *Alpha-* (p = 0.006), *Betaproteobacteria* (p = 0.002) and archaeal (p = 0.004) are significantly affected by increasing sediment depth, likewise the diversity of bacteria (p = 0.008), *Actinobacteria* (p = 0.001) and *Gammaproteobacteria* (p = 0.009) as illustrated by Pearson correlation analysis. Besides, sediment density also exerted weak force in affecting the diversity of *Actinobacteria* (p = 0.091), *Gammaproteobacteria* (p = 0.092) and the community structure of *Betaproteobacteria* (p = 0.06) while

significantly affecting archaeal diversity (p = 0.013). In general, microbial taxa should decrease when sediment depth increases. However, sediments types, density, pore size and pore throat size are often associated with one another and that can result in the restriction of microbial mobility and migration in laminated sediments. Studies indicated that the mobility of microorganisms can be restrained in the sediments with pore sizes equal to or less than a micron and suggested that the pore throat size must be at least twice the diameter of the bacterial cell in order for bacteria to transit (Rebata-Landa and Santamarina, 2006). Besides, sediment pore size could also affect nutrient transportation, space availability for bacteria as well as bacterial division rate, hence the diversity of the microbial population (Fredrickson *et al*., 1997; Rebata-Landa and Santamarina, 2006). Zhang *et al*. (1998) found that grain size has significant correlation with bacterial abundance and activity below the soil zone at the Abbott Pit site. They showed that changes in grain size at centimeter scale could have a predominant effect on microbial variability. In Suigetsu sediment, the mobility of the microbial taxa from younger to older sediment could be vastly restricted due to the compactness and pore size of the laminated sediment, thereby, affected their diversity and community structure.

Table 4.12 Environmental variables noted for the entire Lake Suigetsu sediment cores

Biome	Core	Age (BP)	Depth (cm)	TOC (%)	TN (%)	$\delta^{15}N$	Density (gcm^{-3})	Water co. (%)
WAMX	A (N) 01	567	148.55	3.88	0.51	4.25	0.34	77.85
	A (N) 03	4124	599.53	5.87	0.65	4.52	0.30	74.70
	B (N) 05	6860	932.44	7.21	0.86	4.23	0.27	74.61
	B (N) 07	10911	1363.76	6.51	0.70	4.95	0.30	72.75
COMX /TEDE	B (N) 11	22502	2150.45	2.77	0.40	5.46	0.50	62.59
TEDE	A (S) 13	28911	2510.00	1.81	0.31	3.89	0.58	56.99
	A (N) 16	34945	2930.22	4.35	0.49	4.31	0.56	57.49
	B (N) 17	41635	3359.29	2.66	0.31	3.69	0.77	44.58
	B (N) 19	48376	3761.25	3.33	0.41	3.05	0.67	52.38
UNKNOWN	B (N) 21	60354	4197.10	2.87	0.38	2.96	0.63	52.27
	A (N) 24	74328	4549.76	5.19	0.43	1.67	0.55	59.54
	A (N)28	88842	4998.20	3.88	0.38	3.36	0.54	58.10
	A (S) 30	101916	5400.68	4.47	0.45	2.16	0.47	58.11
	A (S) 32	112998	5799.90	3.24	0.39	1.85	0.59	53.92
	A (S) 35	121274	6199.88	6.58	0.53	0.83	0.36	63.51
	A (N) 39	128993	6549.74	7.92	0.67	-2.43	0.59	50.54
	A (N) 43	136166	6946.65	13.54	0.64	0.13	0.63	44.36
	A (N) 46	143019	7247.69	4.59	0.37	1.11	1.00	38.18

Biome zones indicated according to Gotanda *et al*., 2002. WAMX = warm mixed forest, TEDE = temperate deciduous forest and COMX = cool mixed forest. Age (BP) stated in the average of the 10 cm sample core. This elemental data was obtained from the Suigetsu project website http://kairos.naruto-u.ac.jp/~suigetsu/. Depth: sediment composite depth, TOC: Total organic carbon, $\delta^{15}N$: ratio of stable isotope of nitrogen, Density: sediment density, Water co: water content and TN: Total nitrogen.

Apart from sediment depth and density, total organic carbon (TOC) and total nitrogen (TN) also exerted some forces in shaping the diversity and community structure of bacteria, *Actinobacteria* and *Archaea*. TOC in Suigetsu sediment appeared to slightly influence the diversity of the bacterial community (p = 0.087) and the community structure of *Actinobacteria* (p = 0.096) and *Archaea* (p = 0.082). On the other hand, TN exerted significant effect on *Actinobacteria* diversity (p = 0.015) but weak forces on archaeal community structure (p = 0.080). In fact, nutrient availability is considered as the primary factor that can change the microbial communities in lake sediments (Nam *et al*., 2008). The findings from Nam *et al*. (2008) on Lake Hovsgol showed similar outcomes to our study, in which the diversity of microbial communities is very much lesser in deeper sediment layers compared to the upper layers, so as the trend of nutrient availability (Table 4.12). They suggested that such phenomena are due to the paleoclimatic events however discussion on how climatic events influence both nutrient availability and microbial diversity has not been made.

In fact, the productivity in the meromictic lake will markedly reduce during colder climate, suggesting that nutrient availability should be high in the lake and vice versa (Nam *et al*., 2008). However, this is not the case for our study. Table 4.12 showed the average units of TOC and TN are higher in the WAMX biome zone (5.87; 0.68) than in colder biome of TEDE (3.04; 0.38). Species richness and microbial Shannon diversity also showed positive relationships to TOC and TN. This could reflect that the microbial ecology in lake water is not the same as in the lake sediment. Zhou *et al*. (2004) reported that the communities that are farther from the primary nutrient resource on the top surface sediment therefore may not receive enough labile substrates and the deficit of various carbon sources could result in fewer microbial species. In addition, Rebata-Landa and Santamarina (2006) also indicated that pore size can limit the transportation of nutrients, particularly in the region with small particle sizes. The sediment of Lake Suigetsu is mainly composed of clayish materials and with some sandy layers. Deposition of clay minerals will form denser lamination over time while sandy sediments are less dense. Nitrogen resources in this case could be transported easily through layers of sandy sediment but not the denser clayish sediments of Lake Suigetsu, which may also contribute to fewer microbial diversity and changes in community structure in deeper sediment of Lake Suigetsu. *Actinobacteria*, as mentioned before, are mostly aerobes and the substantial positive effect of TN on *Actinobacteria* diversity may suggest their importance in nitrogen cycles in Lake Suigetsu sediments.

Acidobacteria community appeared to be not affected by any of environmental parameters. *Acidobacteria* in general, favours oligotrophic environments with low level of nutrients and they can withstand drier soil conditions. Another study has also revealed that changes in climatic conditions do not affect the diversity and abundance of *Acidobacteria* (Castro *et al*., 2010). Hence, this may support their ubiquity and highly diversified communities throughout the entire sediments of Lake Suigetsu.

Archaeal populations that have been found in aquatic anoxic sediments are usually methanogens, which means they produce methane as by-products in anoxic conditions (Balch *et al*., 1979, DeLong, 2003; Schleper *et al*., 1997). However, methanogenesis is unlikely to occur in Suigetsu sediment as no methane was detected so far from previous studies. The high level of inorganic sulphate substances contained in Lake Suigetsu can inhibit methanogenesis as methanogens will be outcompeted by sulphate-reducing bacteria (SRB) in environments with high sulphate concentrations

(Loveley and Klug, 1983, 1986). Studies found the major archaeal community that was frequently recovered from freshwater sediments are from the phylum *Crenarchaeota* (Cytryn *et al*., 2000; Maturrano *et al*., 2006; Jiang *et al*., 2007; Jiang *et al*., 2008) in which the phylum *Crenarchaeota* was originally thought to consist of only the extreme thermophiles at low evolutionary rates (Schleper *et al*., 1997). Schleper *et al*. (1997) indicated that the presence of anaerobic but nonthermophilic crenarchaeotal phylotypes in the sediments of an oligotrophic freshwater lake could be more ecologically important than was previously thought. However further identification is necessary to confirm whether the archaeal community is from the phylum *Crenarchaeota*.

Last but not least, $\delta^{15}N$ is a stable isotopic indicator that has been used to trace variations in aquatic nitrogen cycling related to the aquatic primary productivity (Amundson *et al*., 2003; Waser *et al*., 1998; Xu and Jaffé, 2008) such as the information on the vegetation in lakes and their catchments (Wolfe *et al*., 1999). $\delta^{15}N$ appeared to show significant relationship to the *Actinobacteria* diversity ($p = 0.002$), the community structure of *Acidobacteria* ($p = 0.002$) as well as both the community structure and diversity of bacteria ($p = 0.002$; 0.008) and *Gammaproteobacteria* ($p = 0.002$; 0.005). Temperature is one of the controlling factors of the ratio of $\delta^{15}N$, which has been reported to be related to denitrification rate that varies across seasons (Song *et al*., 2011). When climate shifts from colder to warmer episode, the ratio of $\delta^{15}N$ will become higher (Amundson *et al*., 2003; Bertrand *et al*., 2003; Deutsch *et al*., 2010). This is because denitrification causes the preferential loss of isotopically light products of ^{14}N and therefore, in warmer conditions, as denitrification rates increase, the residual nitrate pool will become enriched with the heavy isotope of ^{15}N (Bertrand *et al*., 2003; Meckler *et al*., 2007).

Other studies reported that the relative proportion of $\delta^{15}N$ reflects the presence of active denitrifying prokaryotic communities which have important contributions to denitrification rates (Tyler *et al*., 2010). However, the ratio of $\delta^{15}N$ can also be affected by nitrification or detoxification of high concentrations of ammonium (NH_4^+) in the sediments (Kim *et al*., 1997). Tyler *et al*. (2010) specified that the decomposition of organic matter through deamination processes can also potentially change the ratio of $\delta^{15}N$ in sediments. Similarly, under anoxic environment, nitrate will be reduced to ammonia or assimilate through fermentation of ammonia, instead of being reduced to atmospheric N_2 gases through denitrification (Steingruber *et al*., 2001). In this reaction, nitrate will be used as the terminal electron acceptor by the fungal community which is

important for their growth. In addition, the generally high ratio of $\delta^{15}N$ could also likely be the result of terrestrial soil decomposition supplying ^{15}N-enriched nitrate into the lake (Wolfe *et al*., 1999) and thereby may not reflect high denitrification rates or other microbial activities. Moreover, Matsuyama and Sajio (1971) reported that the amount of ammonium was approximately two-times higher than that of nitrate and nitrite, which contributed to the greater part of the inorganic nitrogen in the deep water layer of Lake Suigetsu. This therefore implies that $\delta^{15}N$ data in this study does not necessarily refer to only nitrate levels in Suigetsu sediments but may be contributed by the levels of ammonia. In this case, the direct correlation of $\delta^{15}N$ ratio to denitrification and climatic change could not be established. Therefore, the identification of microbial community as potential biomarkers for climate change cannot be confidently made.

4.5. Conclusions:

In conclusion, most of the bacterial communities in the deep sediment of Lake Suigetsu were more significantly affected by sediment depth and density than nutrient availability. The biomarker of past climate change was however difficult to be identified although species richness and microbial diversity did correspond to changes in biome. $\delta^{15}N$ ratio appeared to be the potential indicator for climate change, given the fact that temperature is the main controlling factor of denitrification and thus the ratio of $\delta^{15}N$. A study by Saunders and Kalff (2001) reported that denitrification accounted for a large proportion of TN compared to nitrogen sedimentation and aquatic plants uptake. This therefore indicates that the measurement of nitrate in the lake sediment is necessary as it may provide important information in relation to the effect of climate change on microbial community.

Chapter 5 Microbial diversity analysis when lake waters condition changes from freshwater to brackish

5.1. Introduction

Saline environments are considered as extreme habitats for microorganisms (Jiang *et al*., 2007; Litchfield and Gillevet, 2002; Rothschild and Mancinelli, 2001). Saline meromictic lakes for instance are frequently used as a model to study the nutrient cycling of carbon, sulfur and nitrogen under oxic and anoxic conditions (Holmer and Storkholm, 2001; Humayoun *et al*., 2003; Meyers and Eadie, 1993; Nam *et al*., 2008) and to understand microbial diversity and functions in extreme ecosystems (Dong *et al*., 2006; Torsvik and Øvreås, 2002). By investigating the influence of environmental factors on the composition and diversity of microbial communities (Humayoun *et al*., 2003; Jiang *et al*., 2007; Jiang *et al*., 2008; Koizumi *et al*., 2004), microbial functions in biogeochemical processes can be understood (Ohkouchi *et al*., 2005; Torsvik and Øvreås, 2002).

Besides the importance of bacterial communities in saline environments, studies on salinity effects on the composition of microbial communities are also essential to examine the functional performances (i.e. salt tolerance) of the microbial community when salinity in an aquatic environment changes (Langenheder *et al*., 2003). In general, salinity is known to impact on microbial community composition (Oren, 2002; Jiang *et al*., 2006). Previous investigations have largely been carried out on dynamic estuaries (del Giorgio and Bouvier, 2002; Langenheder *et al*., 2003; Zhang *et al*., 2006) and coastal solar salterns (Casamayor *et al*., 2002) to investigate the influence of salinity on the microbial community composition. In addition, the comparison between communities of 32 pristine Tibetan lakes (from freshwater to hypersaline lakes) that represent a broad salinity range have also been explored (Wang *et al*., 2011). Similarly, such technique can be utilised to examine the evolution of salinity environment on meromictic lakes by studying the composition of microbial community in the lake sedimentary records.

Lake Suigetsu has experienced limnological change of freshwaters to a brackish meromictic lake (Kato *et al*., 2004; Matsuyama 1974; Matsuyama and Sajio 1971) due to the tectonic uplift caused by Kanbun earthquake in 1662 AD (Kato *et al*., 2004; Takemura *et al*., 1994). Seawater was introduced into Lake Suigetsu when a canal was built to drain the water to the neighbouring polyhaline Lake Kugushi in 1664 AD. Gradually, with no mixing of lake waters, intruding seawaters sank down to the lake bottom and formed a permanent chemocline between 3 m to 8 m which separated the aerobic freshwater upper layers from the anaerobic saline bottom water (Kondo *et al*., 2000; 2006; Matsuyama, 1973a, 1973b; Matsuyama and Saijo, 1971).

A number of researchers have made use of this opportunity to study the vertical distributions of bacterioplankton communities throughout the entire water column at the oxic zones, chemocline and anoxic regions (Kondo and Butani, 2007; Kondo *et al*., 2009; Okada *et al*., 2007). Most of these microbial ecology studies of Lake Suigetsu have largely focused on the lake water columns and investigations from lake sediments are relatively few (Kondo *et al*., 2006; Kondo *et al*., 2009; Okada *et al*., 2007). There has been no investigation on the composition and diversity of microbial communities of pre- and post-salinity influxes based on Lake Suigetsu sediments. In effect, little is known about the responses of freshwater microbial community to the geochemistry and ecology when salinity increases due to seawater invasion. Therefore, given the opportunity to be able to access to Suigetsu sedimentary records, this study aims to examine the community structure and diversity of bacterial populations before and after seawater intrusion through the molecular approaches of PCR-DGGE and metagenomics sequencing. In addition, we have access to the data on microbial community of the water column (Kondo *et al*., 2009), thus it will be interesting to also compare our bacterial community data from preserved DNA in the sedimentary records to this study (lake waters), aiming to evaluate if the microbial composition in the freshwater and brackish sediments is comparable to those in the freshwater epilimnion and the brackish hypolimnion, respectively.

5.2. Experimental strategy:

Sediment records within core SG06-A01 have recorded information on the seawater incursion that occurred during 1664 AD. Sediment depths from 409 BP to 335 BP at 100.72 cm to 77.6 cm were targeted, covering the freshwater and brackish

regions. A total of 20 sediment samples were taken. Fossil DNA was extracted, followed by PCR-DGGE and bacterial 16S rDNA analysis to study the bacterial community. In addition, eleven out of the 20 sediment samples were chosen for metagenomics sequencing, with five from the brackish regions and six from the freshwater, to enhance the study of salinity effects on ancient bacterial communities at the species level.

5.3. Results:

5.3.1. Confirmation of Repeatable Microbial DGGE Data

First, to test whether or not the microbial data are reproducible, four sediment samples were randomly selected with two from the upper cores (A01-6 and 8) and another two from the deepest depths (A01-51 and 54). Selected sediment samples were extracted in triplicate and analysed using PCR-DGGE. Figure 5.1 shows that the replicated bacterial DGGE profiles of each sediment sample are highly reproducible. This indicated that the results are consistent hence ensured the reproducibility of our data.

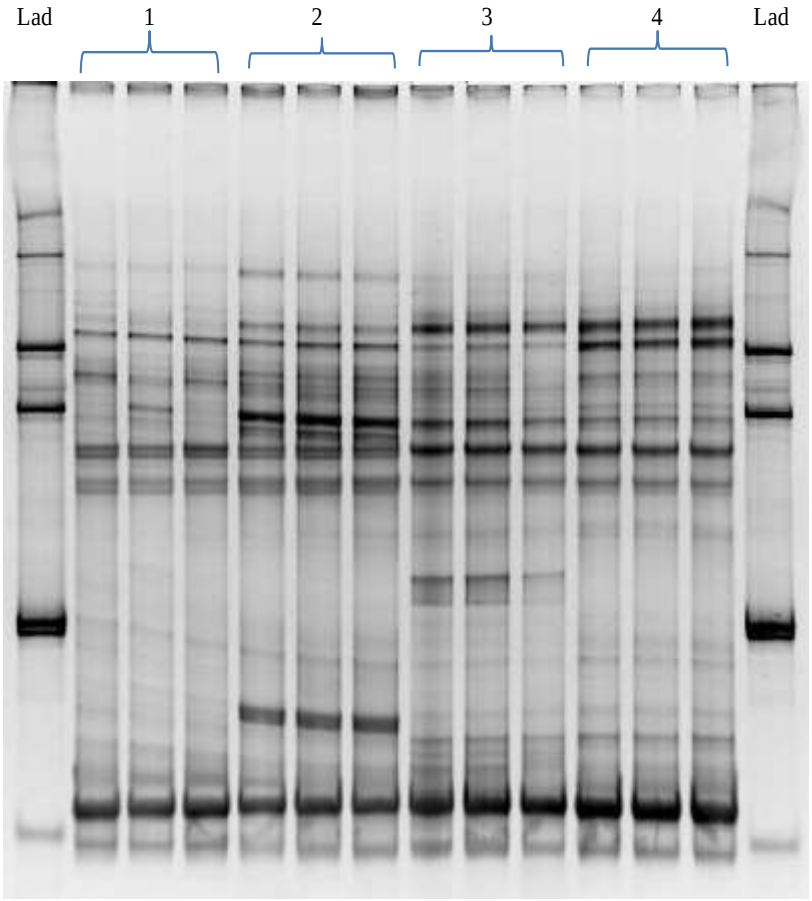


Figure 5.1: Bacterial DGGE profile of sediment sample SG06, 1= A01-6, 2 = A01-8, 3 = A01-51 and 4 = A01-54 in triplicate. This is the bacterial 16S rDNA genes based on DNA analysis. Composite depth of A01-6 = 77.6 cm (351 BP), A01-8 = 84.68 cm (357BP), A01-51= 96.58 cm (396 BP) and A01-54 = 99.68 cm (405 BP). Lad = ladder.

5.3.2. DGGE Analysis of Bacterial Community before and after Salinity Influx

DGGE fingerprint profiles of the bacterial community before and after seawater intrusion were produced (Figure 5.2). Lanes 1 to 5 represent sediment samples after seawater incursion while lanes 6 to 20 correspond to freshwater sediment samples. The bacterial DGGE fingerprinting revealed that both the brackish and freshwater zones contained highly diverse bacterial communities. Species richness and Shannon Weiner Index were also calculated for each of the bacterial DGGE profiles as shown in Table 5.1. These analyses showed that the diversity of bacterial populations in the brackish sediments (mean of Rr = 28; mean of *H*' =2.725) is similar to the freshwater sediments' (mean of Rr = 27.8; mean of *H*' =2.891).

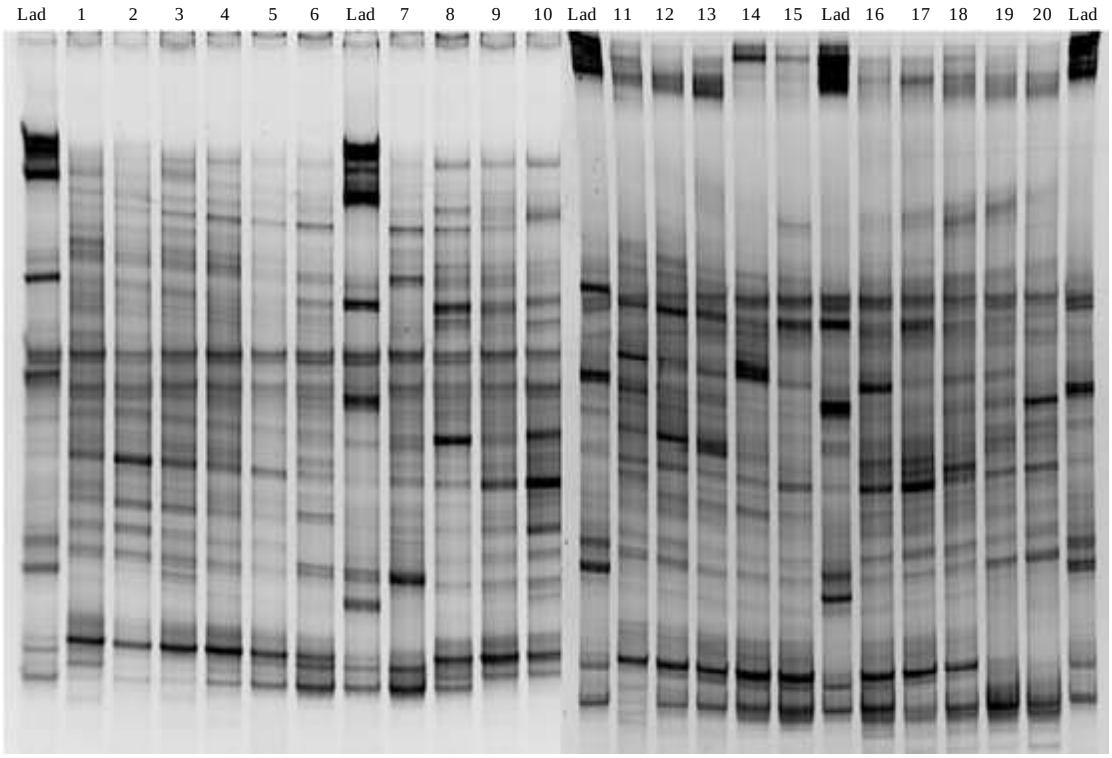


Figure 5.2: DGGE profile of bacterial 16S rDNA genes based on DNA analysis. Lanes 1-5 represent the brackish sediments from depth 77.60 cm to 81.64 cm (335 BP to 348 BP). Lanes 6-20 fall in the freshwater region from depth 82.66 cm to 100.72 cm (351 BP to 409 BP).

Table 5.1: Characterisation of prokaryotic community profiles based on species richness and diversity within freshwater and saline regions of Lake Suigetsu sediment cores

Condition	Core SG06-A01	Age (BP)	Composite depth (cm), mean	Bacterial DGGE Profile	
				Rr (Species richness)	*H*' (Shannon-Wiener Index)
Brackish	1	335	77.60	30	1.655
	2	338	78.61	30	3.088
	3	341	79.62	27	2.924
	4	344	80.63	27	2.964
	5	348	81.64	26	2.995
			Mean	28	2.725
Freshwater	6	351	82.66	27	2.712
	7	354	83.67	30	2.875
	8	357	84.68	30	2.664
	9	361	85.69	29	2.946
	10	364	86.70	26	2.944
	11	379	91.40	30	3.026
	12	382	92.44	26	2.911
	13	386	93.47	26	2.812
	14	389	94.50	29	2.836
	15	392	95.54	29	2.877
	16	396	96.58	31	2.992
	17	399	97.61	28	3.052
	18	402	98.64	28	3.038
	19	405	99.68	23	2.807
	20	409	100.72	25	2.878
			Mean	27.8	2.891

Composite depth is taken from the midpoint of each of the 1 cm^3 sediment sample. Each sediment age was measured using Level Finder Software 4.6.1. Each sediment age represents the age at the midpoint of the composite depth. Rr = species richness. Rr was calculated from each DGGE lane. *H*' = Shannon Weiner Index of diversity. *H*' was calculated using ln *x* (natural logarithm) of the normalised DGGE data; i.e. ln (*x*)*x.

5.3.3. PCA analysis

Principle component analysis (PCA) analysis was first conducted to identify the variance of the bacterial communities' structure across the salinity shift. Figure 5.3a showed two obvious clusters in which the sediment samples from the top of the core were separated from samples from deeper sediments. Both PC1 and PC2 showed a relatively low total variance of 27.2% which indicated a weak support of salinity effect on the bacterial community. Figure 5.3b showed that the topmost microbial communities of brackish sediment samples 1 and 2 were separated along PC1 axis which explained a total variance of 35% in comparison to the microbial communities of

sediment samples 3, 4 and 5 which were affected by PC2 as explained by a total variance of 26.1%. For freshwater sediment samples, the principles components did show a large total variance of 27.2%, suggesting that there was an effect on the population as it transitioned from fresh to brackish water (Figure 5.3c).

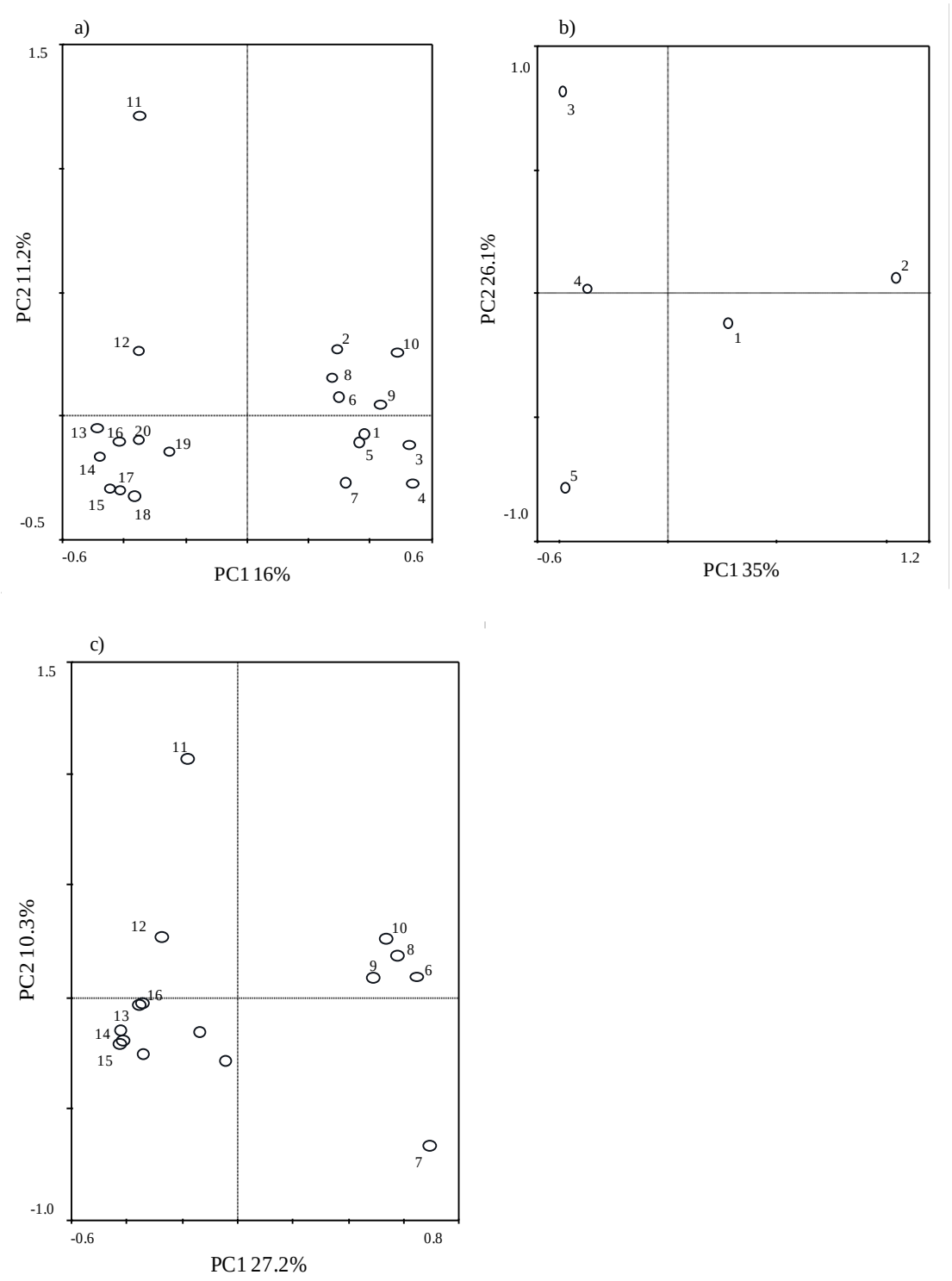


Figure 5.3: a) bacterial community in sediment samples before and after salinity shift, b) bacterial community from brackish sediments, c) bacterial community from freshwater sediments. Sediment samples 1-5 = brackish, 6-20 = freshwater.

5.3.4. Raup-Crick Similarity index

Raup and Crick Similarity Index analysis (Table 5.2) was also calculated for both conditions to measure if the similarities between bacterial community structures were significantly higher or lower than would be observed by chance (Baxter and Cummings, 2006; Rowan *et al.*, 2003). This analysis demonstrated that within the brackish sediment samples on the upper layers, there were five bacterial community profiles which were significantly similar and related to each other. Besides, significant similarities between bacterial community profiles were also observed within the freshwater sediment samples, from samples 11 to 20 in the deeper depths. However, when the bacterial community profiles of the brackish sediments were compared to the freshwater, most of the profiles showed no significant similarity to each other.

5.3.5. Multivariate analysis of microbial communities' structure

Multivariate analysis of RDA was performed as the axis length was calculated to be less than 3.5. RDA was employed to determine which environmental factors had significantly affected the bacterial community structure. Seemingly, only sediment depth appeared to have significant effect (p = 0.002) in changing the bacterial community structures across different sediment conditions (Figure 5.4a, Table 5.3) and within the freshwater zone (p = 0.018) (Figure 5.4c, Table 5.3).

Table 5.2: Raup-Crick Similarity Index Analysis of Bacterial Communities across Salinity Shifts

	1	**2**	**3**	**4**	**5**	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		**0.99**	0.94	**1.00**	1.00	0.94	0.27	0.77	0.79	0.92	0.23	0.80	0.46	0.18	0.25	0.18	0.42	0.79	0.18	0.19
2			0.85	0.87	**0.96**	0.18	0.64	0.77	0.80	0.91	0.11	0.40	0.92	0.94	0.24	0.21	0.25	0.90	0.74	0.32
3				**0.99**	0.92	0.60	0.53	0.54	**0.95**	0.93	0.33	0.71	0.52	0.23	0.33	0.79	0.86	0.70	0.22	0.10
4					**1.00**	0.79	0.51	**0.95**	**0.95**	0.94	0.03	0.51	0.50	0.12	0.17	0.28	0.70	0.85	0.08	0.23
5						0.83	0.43	0.80	**0.99**	**1.00**	0.66	0.62	0.89	0.50	0.63	0.33	0.60	0.60	0.26	0.50
6							0.54	0.94	0.54	0.49	0.54	0.32	0.15	0.12	0.18	0.65	0.31	0.30	0.10	0.10
7								**1.00**	**1.00**	0.91	**0.00**	**0.05**	0.25	0.19	0.12	0.31	0.14	0.26	0.55	0.19
8									**1.00**	0.93	0.43	0.27	0.65	0.09	0.26	0.53	0.41	0.25	0.19	0.51
9										**1.00**	0.41	0.25	0.64	0.19	0.43	0.53	0.24	0.24	0.53	0.18
10											0.63	0.61	**0.96**	0.73	0.44	0.19	0.43	0.39	0.81	0.68
11												**1.00**	**0.97**	**0.98**	**0.99**	**0.98**	0.79	0.63	0.56	0.94
12													**1.00**	**1.00**	**1.00**	**0.98**	**0.99**	0.91	0.82	**0.98**
13														**1.00**	**1.00**	**0.99**	**1.00**	**0.97**	**1.00**	**1.00**
14															**1.00**	**1.00**	**1.00**	**0.99**	**0.97**	0.90
15																**1.00**	**1.00**	**1.00**	**0.95**	**0.99**
16																	**1.00**	**1.00**	0.81	**0.98**
17																		**1.00**	**0.99**	**1.00**
18																			**1.00**	**1.00**
19																				**1.00**
20																				

Codes 1 – 5 (in bold) = samples from brackish sediments, 6 – 20 = samples from freshwater sediments. The underlined value implies = dissimilarity ($S_{RC} < 0.05$), the highlighted value in red = significant similarity ($S_{RC} > 0.95$) and the rest = similarity occurred by chance ($0.05 < S_{RC} < 0.95$).

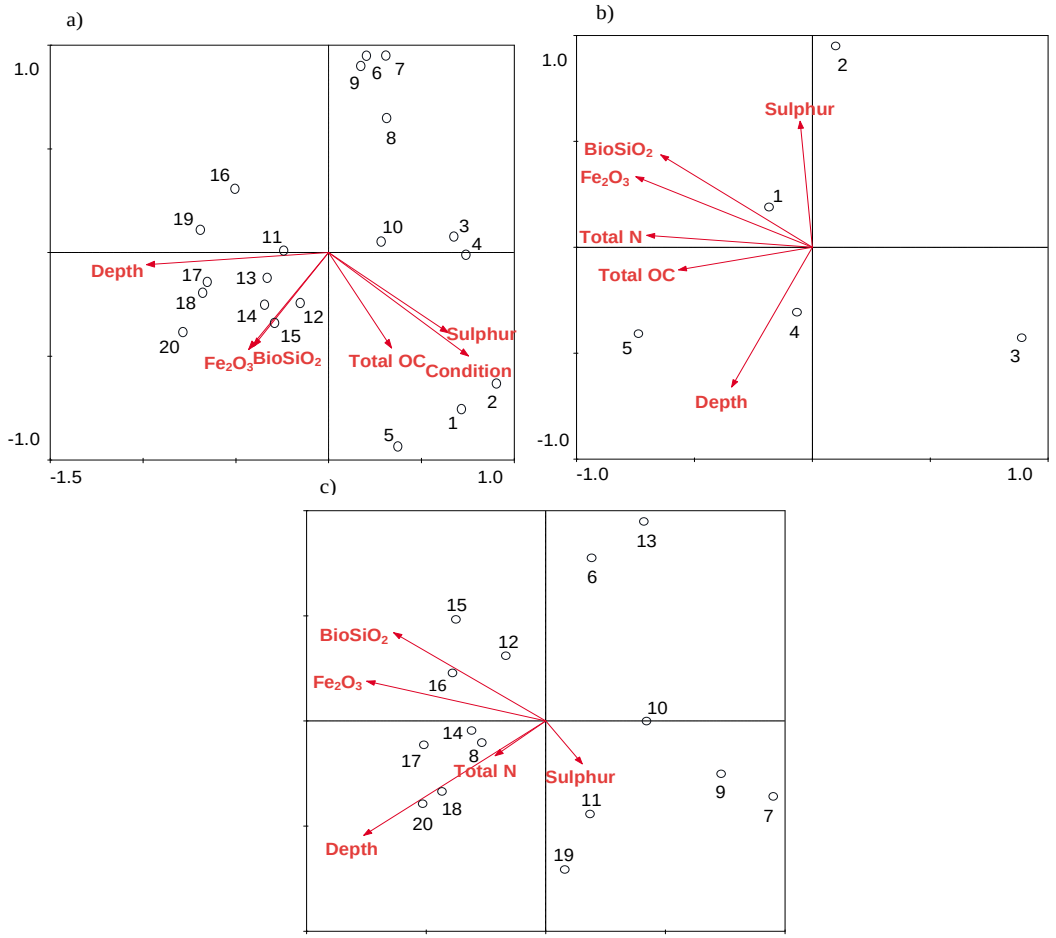


Figure 5.4: RDA analysis of bacterial communities' structures a) across salinity shift; b) brackish region; c) freshwater region. Sediment sample 1-5 = brackish; 6-20 = freshwater. Depth = sediment composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3= Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$= biogenic silica oxide, condition = brackish/freshwater condition.

Table 5.3: Significant evaluation of environmental factors against bacterial community structures based on RDA Analysis

	Conditional Effects		
	Freshwater - Brackish	**Brackish**	**Freshwater**
Variable	*p*	*p*	*p*
Depth	**0.002**	0.462	**0.018**
Total OC	0.926	-	-
Total N	0.692	1	0.506
Fe_2O_3	0.896	0.148	0.846
Sulphur	0.51	-	0.344
$BioSiO_2$	0.304	0.442	0.204
Condition	0.224	-	-

Highlighted *p* in red represents significance value. $p < 0.05$ is considered to have significant correlation between bacterial community structures and environmental factors while $p > 0.05$ would be less significant. Depth = composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3= Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$= biogenic silica oxide, Condition = brackish/freshwater condition. Those with no input were excluded in the analysis as inflation factor (IF) is higher than 20.

5.3.6. Pearson correlation coefficient analysis of microbial diversities

Pearson correlation was further applied to investigate environmental factors against bacterial diversity. This analysis however showed no significant correlation between bacterial diversity and environmental factors (Table 5.4).

Table 5.4: Significant evaluation of environmental factors against bacterial diversity based on Pearson Correlation

Variables		**Freshwater - Brackish**	**Brackish**	**Freshwater**
Depth	**Coef.**	0.326	0.672	0.411
	p	0.160	0.214	0.128
Salinity	**Coef.**	-0.244	-	-
	p	0.299	-	-
Total N	**Coef.**	0.208	-0.123	-0.486
	p	0.423	0.844	0.109
Total OC	**Coef.**	-0.396	-0.724	-0.316
	p	0.115	0.167	0.318
Sulphur	**Coef.**	0.025	0.709	-0.247
	p	0.928	0.180	0.464
$BioSiO_2$	**Coef.**	-3.58	-0.941	-0.267
	p	0.1783	0.219	0.378
Fe_2O_3	**Coef.**	0.282	0.674	-0.114
	p	0.289	0.529	0.711

Table above shows relationship between bacterial diversity and environmental factors. Depth = composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3= Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$= biogenic silica oxide, Condition = brackish/freshwater condition. Coef. = Pearson Correlation coefficients and *p* = p value. p values ≤ 0.05 = significant correlation. A positive coefficient value = positive relationship and negative coef. value = negative correlation. (-) = no comparison made.

5.3.7. Comparison between DGGE and Metagenomics Analysis on Bacterial Communities before and after Salinity Influx

Sediment samples of SG06 A01- 1 to 7, 12a, 12b, 19 and 20 were selected for metagenomics analysis (Table 5.5). Sediment samples 12a and 12b were the 1 mm sediment slices initially obtained for higher resolution studies. These samples originated from the freshwater sediments at depth 88.23 cm (369 BP) and 89.97 cm (374 BP) and they were also included to improve the resolution for the analysis of freshwater bacterial communities in metagenomics study.

Table 5.5 Sediment samples selected for metagenomics analysis

SG06	Sample code	Condition	Composite depth	Age (BP)
A01	1	3	77.60	335
A01	2	3	78.61	338
A01	3	3	79.62	341
A01	4	3	80.63	344
A01	5	3	81.64	348
A01	6	3	82.66	351
A01	7	1	83.67	354
A01	12a	1	88.23	369
A01	12b	1	89.97	374
A01	19	1	99.68	405
A01	20	1	100.72	409

Condition 3 = sediment samples taken from the saline region.
Condition 1 = sediment samples taken from the freshwater region

The DGGE matching data for these samples were pulled together and re-analysed using PCA analysis (Figure 5.5a and b). Figure 5.5a showed that sediment samples (1-6) from the upper section were separated from sediment samples (7-20) at lower depths (Figure 5.5a).

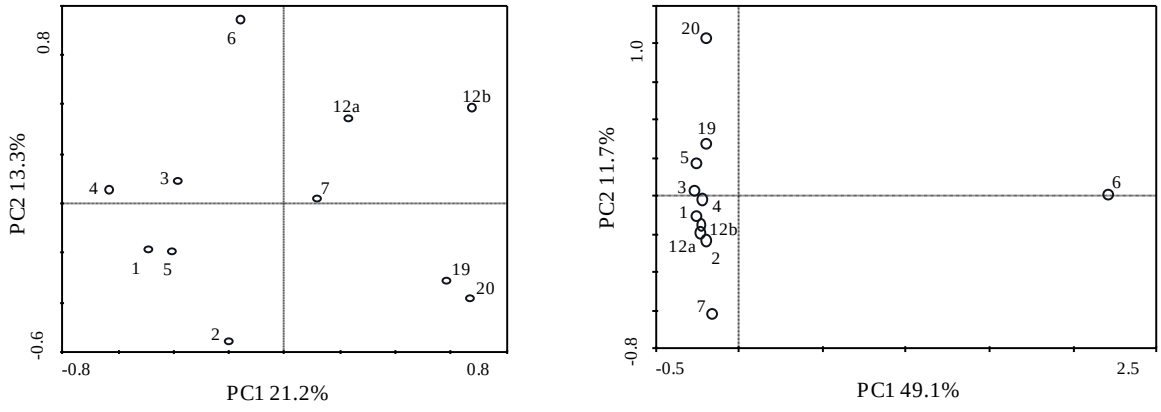


Figure 5.5: PCA analysis of sediment samples from a) DGGE and b) metagenomics data. Sediment samples 1 to 5 = brackish and 6, 7, 12a, 12b, 19, 20 = freshwater.

However, the total variance of 34.5% indicated that the effect of salinity on bacterial community was rather weak. In contrast, PCA analysis of metagenomics data revealed very different distribution pattern of bacterial communities. Sediment sample 6 was strongly affected by PC1 which accounted for a total variance of 49.1% while the rest of the communities were distributed along PC2 as explained by a total variance of 11.7%. In addition, the bacterial communities from above and below sediment sample 6 seemed to be more similar to each other as they were clustered together. On the other hand, metagenomics data showed that the bacterial community of sediment sample 6 was separated far apart from the rest of the bacterial communities, indicating that the bacterial community structure is very distinctive (Figure 5.5b). This observation is however different from our speculation as indicated by LevelFinder software 4.6.1, the event of seawater incursion occurred in 1664 AD should be recorded within depth 81.64 cm, in sediment sample 5.

RDA/CCA analysis was also carried out on the DGGE and metagenomic bacterial community data. RDA analysis disclosed that the community structures of bacterial populations from DGGE data were significantly affected by salinity (p = 0.002) (Figure 5.6a, Table 5.6). Whereas CCA analysis of metagenomics data showed that although salinity was a driver for bacterial community structure (p = 0.098), the total organic carbon content (p = 0.024) in the lake sediment was the most significant factor affecting them (Figure 5.6b, Table 5.6).

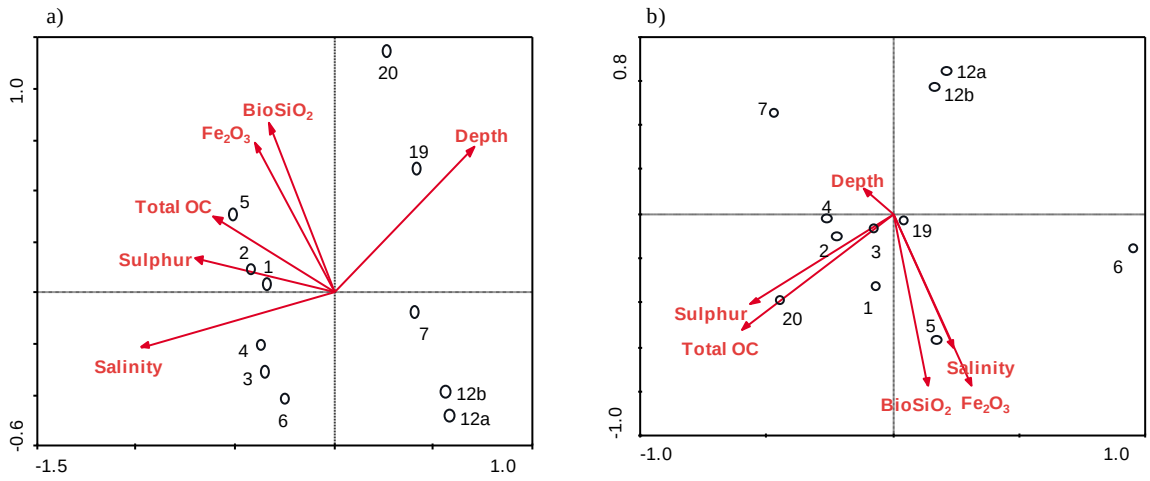


Figure 5.6: a) RDA analysis of bacterial community based on DGGE data. b) CCA analysis based on OTU measurements of metagenomics data. Sediment samples 1 to 5 = brackish, 6, 7, 12a, 12b, 19, 20 = freshwater. Depth = sediment composite depth, Total OC = total organic carbon, Fe_2O_3= Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$ = biogenic silica oxide, Salinity = brackish/freshwater condition

Table 5.6: Significant analysis of bacterial community structure against environmental parameters

Variable	Conditional Effects	
	DGGE	Meta
	p	*p*
Total OC	0.87	**0.024**
Fe_2O_3	0.44	0.634
Sulphur	0.496	0.252
$BioSiO_2$	0.924	0.78
Salinity	**0.002**	*0.098
Depth	0.66	0.23

Highlighted *p* values < 0.05 = significant correlation. * = weak correlation. Depth = sediment composite depth, Total OC = total organic carbon, Fe_2O_3= Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$= biogenic silica oxide, Salinity = brackish/freshwater condition.

Furthermore, a stacked bar chart based on the OTU counts of metagenomics analysis at order level showed that the bacterial taxa in brackish sediments are very similar to those in freshwater sediments given that *Bacillales* and *Clostridiales* are the major constituents (Figure 5.7, Table 5.7). Taxa that can readily distinguish freshwater bacteria from brackish are *Thermoanaerobacterales* as they are highly abundant in freshwater sediments compared to brackish. In addition, sediment sample 6 also appeared to be very distinctive as it contains great bacterial diversity in which most of the taxa were not found in either freshwater or brackish sediments. This implies that sediment sample 6 is very likely to contain information in relation to the seawater incursion event.

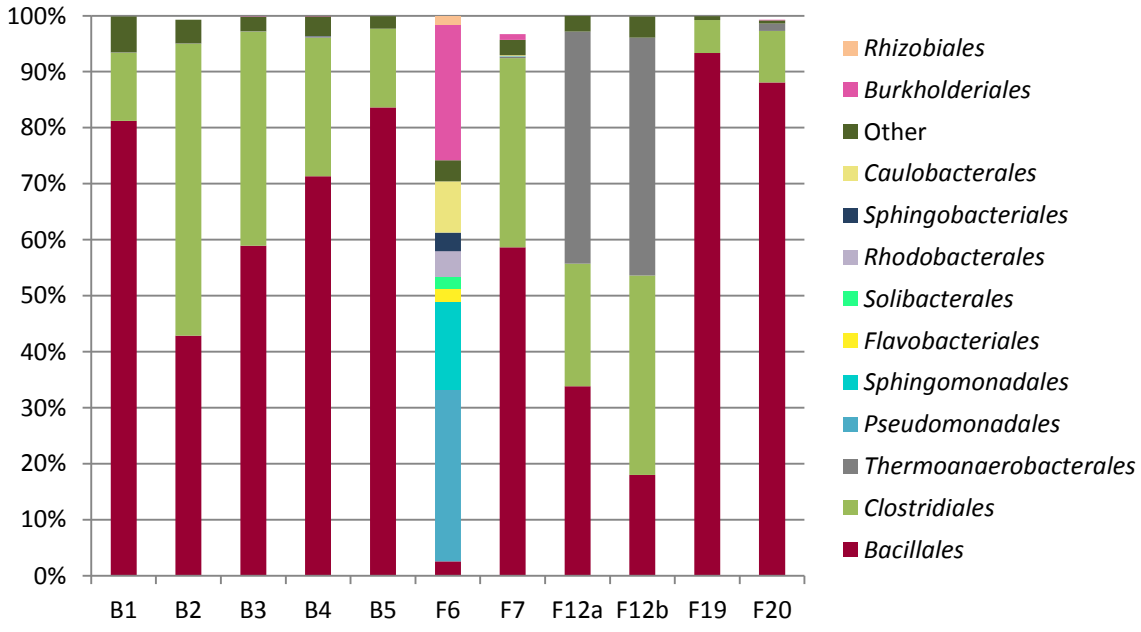


Figure 5.7: Bar chart based on metagenomics data shows taxon at order level in different environmental conditions. F = freshwater; B = brackish. B1 to B5 are from composite depth 77.6 cm – 82.66 cm (335 BP – 351 BP), F6 to F7 = 83.67 cm - 88.23 cm (354 BP - 369 BP), F12a = 88.23 cm (369 BP), F12b = 89.97 cm (374 BP), F19 to F20 = 99.68 cm - 100.72 cm (405 BP - 409 BP). Taxa with OTU counts <1.5 % are grouped as 'Other'.

Table 5.7: OTU counts (%) of taxa at order level in different water conditions

	OTU counts (%)										
	Brackish					Freshwater					
Taxon	**B1**	**B2**	**B3**	**B4**	**B5**	**F6**	**F7**	**F12a**	**F12b**	**F19**	**F20**
Rhizobiales	0.00	0.00	0.00	0.00	0.00	1.55	0.00	0.00	0.00	0.00	0.00
Burkholderiales	0.00	0.00	0.05	0.02	0.00	24.24	1.05	0.00	0.00	0.00	0.10
Others	6.43	4.25	2.56	3.49	2.24	3.81	2.71	2.80	3.76	0.71	0.60
Caulobacterales	0.00	0.00	0.00	0.00	0.00	9.16	0.21	0.00	0.00	0.00	0.00
Sphingobacteriales	0.00	0.00	0.00	0.04	0.00	3.31	0.00	0.00	0.00	0.00	0.00
Rhodobacterales	0.00	0.00	0.00	0.00	0.00	4.51	0.00	0.00	0.00	0.00	0.00
Solibacterales	0.00	0.00	0.00	0.00	0.00	2.26	0.00	0.00	0.00	0.00	0.00
Flavobacteriales	0.00	0.00	0.00	0.00	0.00	2.26	0.00	0.00	0.00	0.00	0.00
Sphingomonadales	0.00	0.00	0.00	0.00	0.00	15.72	0.00	0.00	0.00	0.00	0.00
Pseudomonadales	0.00	0.00	0.00	0.00	0.00	30.66	0.09	0.00	0.00	0.00	0.00
Thermoanaerobacterales	0.05	0.03	0.08	0.25	0.00	0.00	0.21	41.42	42.46	0.00	1.29
Clostridiales	12.18	52.11	38.27	24.76	14.07	0.00	33.79	21.92	35.62	5.90	9.18
Bacillales	81.20	42.89	58.91	71.30	83.59	2.54	58.66	33.83	18.01	93.32	88.10

5.3.7.1. PLS-DA analysis of bacterial communities

Partial least square discriminant analysis (PLS-DA), a regression extension of PCA was also carried out in order to improve and maximise the separation between taxa that correlate to different water conditions, as shown in Figure 5.8 and 5.9. All the sediment samples selected for metagenomics sequencing fell inside the ellipse, indicating 95% confidence level, as defined by Hotellings T^2 *t*-test (Figure 5.8). Figure 5.8 showed a notable grouping of sediment samples from the same former water condition, indicating similar bacterial community structure. PLS-DA loading plot (Figure 5.9) on the other hand, demonstrated the correlation between defined taxa and the sediment conditions. Taxa that were closely distributed to the variable are highly associated to the condition while those separated a distance away would be less similar. PLS-DA analysis at family level also demonstrated that both the freshwater and brackish sediments are dominated by *Clostridiaceae* and *Bacillaceae*. However, *Clostridiaceae* and *Bacillaceae* collectively are more abundant in the brackish sediments as compared to the freshwater sediments. Sediment sample 6 was labelled as 'M' for 'mixing' for PLS-DA analysis as it showed great diversity in previous analyses. Interestingly, sediment sample 6 appeared to have a diverse and unique bacterial composition which comprised of taxa *Oxalobacteraceae*, *Comamonadaceae*, *Sphingomonadaceae*, *Rhodobacteraceae*, *Caulobacteraceae*, *Moraxellaceae* and *Flavobacteriaceae* which are distinctive from those related to freshwater and brackish conditions.

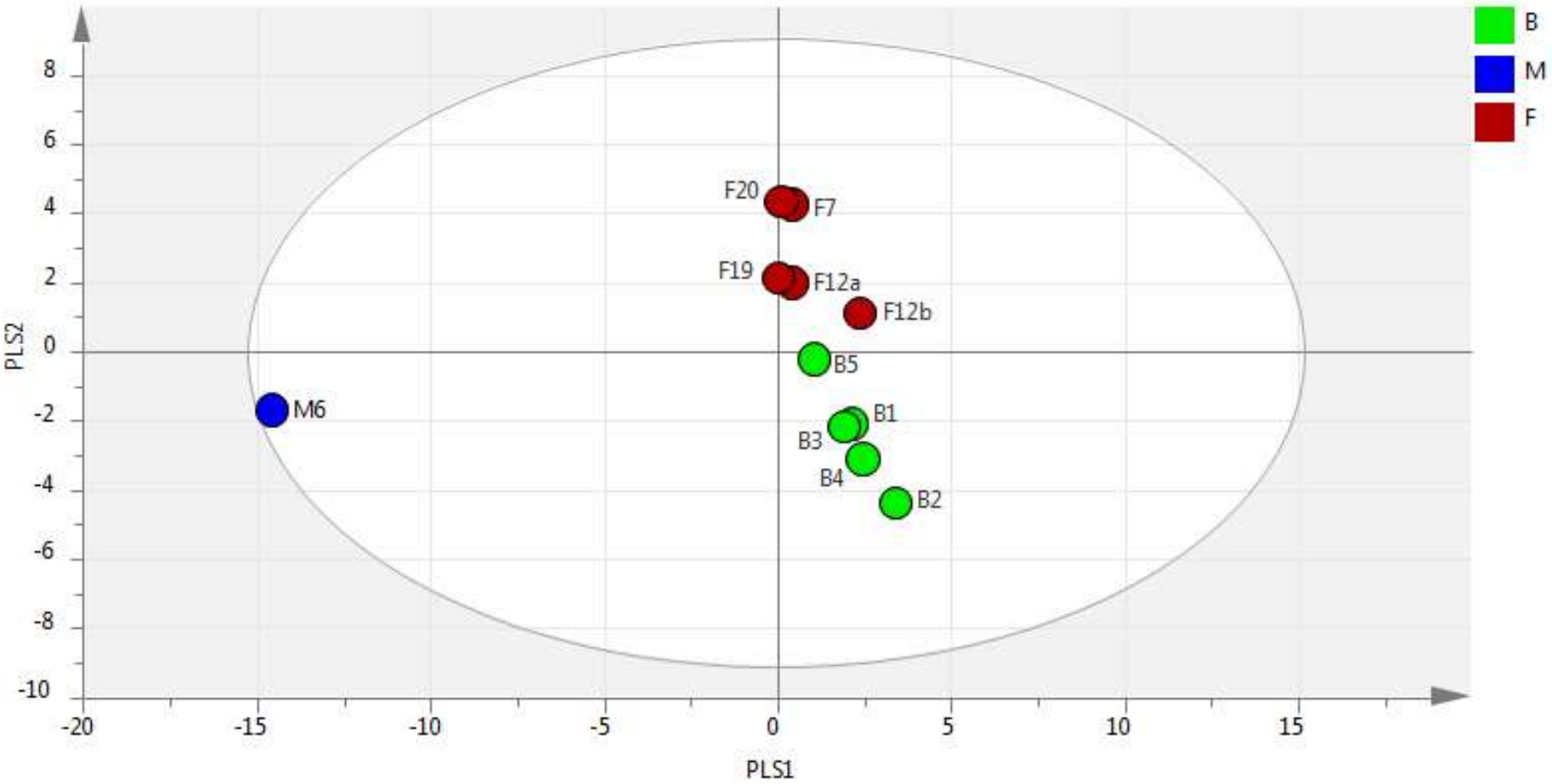


Figure 5.8: PLS-DA based on pyrosequencing data across salinity shifts. The ellipse denotes the 95% significance limit of the model, as defined by Hotelling's *t*-test. F = freshwater; M = mixing and B = brackish.

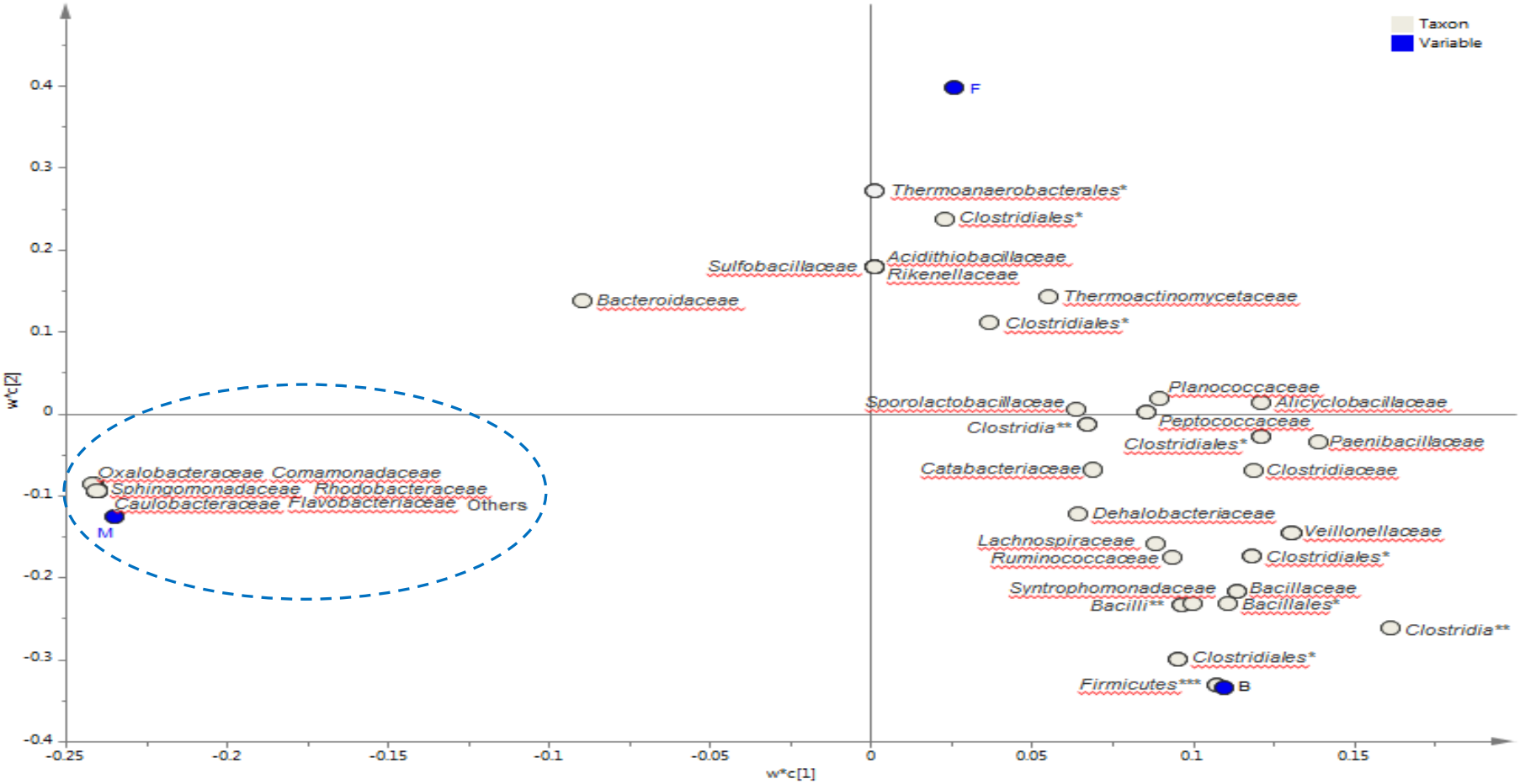


Figure 5.9: PLS-DA loading plot shows the relationship between taxa and environmental variables F, M and B. F = freshwater; M = mixing and B = brackish. Taxa that situated in the area close to the environmental variables suggest significant correlation while taxa located further from the variables indicate weaker relationship. Taxon is described at family level. * is assigned to taxon that has insufficient information to be called at family level. Taxon* = order level, Taxon** = class level, Taxon*** = phylum level. Taxa with OTU counts <1.5 % are grouped as 'Others'. Dotted circle = taxa strictly found in that condition.

5.4. Discussion:

In Lake Suigetsu, sulphide has been reported to be detectable only at the chemocline with increasing concentrations as depth increases and it is the major compound that distinguishes the freshwater epilimnion from the brackish hypolimnion (Mori *et al*., 2013). The salinity of the lake water is generally measured and the epilimnic water of Lake Suigetsu collected in 2005/2006 was 2–4 practical salinity units (psu) while 12–14 psu for the hypolimnic water (Kondo and Butani, 2007). In addition, studies also found that the level of sulphate is usually higher in saline waters (Nielsen *et al*., 2003; Santoro *et al*., 2006) and following the general ecological principles, microbial diversity should decrease in this environment (Frontier, 1985). However, in our study, bacterial diversity did not show similar trend as the average Shannon diversity measured from the brackish sediments (mean H' = 2.725) is nearly as high as that calculated from the freshwater regions (mean H' = 2.828), likewise the species richness. Although it is generally accepted that biodiversity decreases with increasing salinity, the relationship between salinity and biodiversity is not simply a negative correlation when it comes to community-level study (James *et al*., 2003).

Previous studies on the salinity effects on the microbial community composition based on estuaries (Bouvier and del Giorgio, 2002; Langenheder *et al*., 2003, Zhang *et al*., 2006), coastal solar salterns (Casamayor *et al*., 2002) and lake waters (Wang *et al*., 2011) have indicated that salinity is the major factor in controlling microbial abundance, diversity, composition and functions. However, other studies stated that nutrient concentration is also as important (Bouvier *et al*., 2002; Hobbie, 1988). Our DGGE data revealed that sediment depth has significant impact on bacterial community structure in freshwater sediments (p = 0.018) so as across the salinity shift (p = 0.002). RDA analysis of selected DGGE samples (1 – 7, 12a, 12b, 19, 20) showed that salinity has significant effect on bacterial community structure (p = 0.002). CCA analysis based on metagenomics OTU data on the other hand, demonstrated that salinity had only slight impact (p = 0.098) but TOC appeared to be a significant factor in altering the community structures of the bacterial populations (p = 0.024). According to Nielsen *et al*. (2003), the changes in salinity can affect freshwater ecology directly or indirectly and salinisation of a freshwater body can potentially change both the light climate and the mixing properties, which in turn have an impact on the cycling of energy and nutrients. Jiang *et al*. (2007) who also compared both the salinity and nutrient effects on

microbial community indicated that salinity is the primary factor if microbial diversity follows the general ecological principles, which is lowest diversity when highest salinity. Apparently, this is not the case in our study as bacterial diversity, revealed by Pearson correlation, did not appear to be significantly related to any of the environmental parameters. This may therefore suggest that seawater incursion may be the major cause in altering the water chemistry and TOC concentrations in Lake Suigetsu and hence the composition of bacterial community.

Table 5.8: Geochemical analysis of saline and freshwater sediment samples from Lake Suigetsu cores

Core SG06-A01	Age (BP)	Condition	Composite depth (cm)	Total N (%)	Total OC (%)	Sulphur (%)	$BioSiO_2$ (%)	Fe_2O_3 (%)
1	335		77.60	-	5.23	1.41	33.60	4.81
2	338		78.61	0.49	3.71	2.40	21.00	5.89
3	341	3	79.62	0.46	3.84	1.62	-	-
4	344		80.63	0.44	3.54	1.95	-	-
5	348		81.64	0.55	4.88	1.81	25.97	8.68
6	351		82.66	-	-	-	23.11	7.12
7	354		83.67	0.50	4.49	1.60	-	-
8	357		84.68	0.51	4.56	-	35.33	4.96
9	361		85.69	0.52	4.26	1.46	-	-
10	364		86.70	0.46	3.81	1.51	32.28	4.51
11	379		91.40	0.55	4.01	1.16	24.84	5.83
12	382		92.44	0.48	3.87	1.08	36.53	4.97
13	386	1	93.47	0.49	3.81	1.14	27.12	5.96
14	389		94.50	0.47	3.54	1.07	32.17	6.18
15	392		95.54	0.46	3.06	0.80	39.94	4.70
16	396		96.58	-	-	-	24.51	5.59
17	399		97.61	0.41	3.06	0.69	25.52	6.12
18	402		98.64	0.41	2.99	0.77	25.39	5.99
19	405		99.68	-	-	-	26.25	6.15
20	409		100.72	0.43	3.25	0.78	28.61	5.99

Condition 3 = brackish and 1 = freshwater. Composite depth is taken from the midpoint of each of the $1cm^3$ sediment sample. This elemental data was obtained from the Suigetsu project website http://kairos.naruto-u.ac.jp/~suigetsu/. Each sediment age represents the age at the midpoint of the composite depth. Total N = total nitrogen, Total OC = total organic carbon, $BioSiO_2$ = biogenic silicon dioxide (Silica), Fe_2O_3 = Iron (III) oxide/ferric oxide. '-' indicates data unavailable.

In addition, the bar chart in Figure 5.8 also showed that the taxa detected before and after salinity influx are predominated by the same taxa at order level, which are the *Clostridiales* and *Bacillales* from phylum *Firmicutes*. Similarly, PLS-DA analysis revealed that *Clostridiaceae* and *Bacillaceae* at family level, also showed predominance in both freshwater and brackish sediments. Jiang *et al*. (2007) who utilised PLFA

analysis to investigate salinity gradient on the microbial community composition in hypersaline lake sediments also observed that *Firmicutes*, anaerobic low G+C Gram-positive bacteria were present throughout the sediment cores. *Firmicutes* are in fact freshwater and halotolerant organisms that have been shown to predominate in low-salinity sediments, including the deep-sea sediments (Li *et al*., 1999b) and meromictic lakes of marine salinity from Eastern Antarctic (Bowman *et al*., 2000). *Firmicutes* have also been reported to be absent from high-salinity sediments such as the hypersaline environments (Benlloch *et al*., 2002; Demergasso *et al*., 2004). Despite the predominance of *Clostridiaceae* and *Bacillaceae* in both respective conditions, our observation showed that *Clostridiaceae* and *Bacillaceae* are highly abundant in the brackish sediments than in the freshwater. The increased in *Clostridiaceae* and *Bacillaceae* in the brackish sediments may be the result of seawater incursion into Lake Suigetsu and thus adding additional *Firmicutes* group to the lake.

Besides *Clostridiaceae* and *Bacillaceae*, the other dominated groups of taxa in the conditions were different. The dominating groups detected in the brackish sediments all belong to phylum *Firmicutes*, such as *Ruminococcaceae*, *Syntrophomonadaceae*, *Lachnospiraceae* and *Veillonellaceae*, while the predominant groups of freshwater sediments fall into several phyla. These include *Rikenellaceae* from phylum *Bacteroidetes*, *Acidithiobacillaceae* from phylum *Proteobacteria* and *Sulfobacillaceae*, *Thermoactinomycetaceae* and *Thermoanaerobacterales* from phylum *Firmicutes*. Nevertheless, previous studies on inland waters (Glockner *et al*., 1999; Böckelmanna *et al*., 2000; Brümmer *et al*., 2000), estuaries (Crump *et al*., 1999; Bouvier and del Giorgio, 2002; del Giorgio and Bouvier, 2002; Zhang *et al*., 2006), and coastal solar salterns (Benlloch *et al*., 2002) have commonly reported that the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* increases with salinity whereas that of *Betaproteobacteria* decreases. This is however inconsistent with our findings. Jiang *et al*. (2007) who investigated the hypersaline lake sediments did not obtain the similar results as well. Studies indicated that environments such as estuaries and coastal solar salterns are dynamic ecosystems usually with pronounced salinity gradients which are highly productive and commonly exhibit very high rates of bacterial activity compared to the closed basins (Casamayor *et al*., 2002; Findlay *et al*., 1991; Langenheder *et al*., 2003; Selje and Simon, 2003; Zhang *et al*., 2006). Furthermore, the composition of particle-attached and free-living bacterial community can be fundamentally different in terms of responses to salinity and biogeochemical in lake ecosystems (Crump *et al*.,

1999; Phillips *et al*., 1999; Schweitzer *et al*., 2001). Therefore, the salinity effect on the bacterial diversity and community structure in these environments may be different to our study.

Furthermore, the predominance of taxa before and after salinity influx is evidently different. Sediment sample 6 showed highly diverse and distinctive taxa compared to both freshwater and brackish sediments with the absence of *Clostridiales* and minor group of *Bacillales* (2.54%) (Table 5.7). This phenomenon is likely to suggest that there could have been a dynamic mixing of water body due to the seawater incursion. Studies have in fact indicated that dynamic mixing of water bodies can lead to cell stress, loss of activity, as well as cell death and two types of succession such as activation/inactivation and replacement are also likely to occur simultaneously, determining bacterial succession and community composition (Bouvier and del Giorgio, 2002; del Giorgio and Bouvier, 2002). Similarly, Nielsen *et al*. (2003) indicated that changes in salinity can affect freshwater ecology directly or indirectly. The direct effect of increasing in salinity can result in physiological changes resulting in the loss or gain of species. Indirect changes can modify community structure and function by removing (or adding) taxa that provide protection, food or predation pressure (Nielsen *et al*., 2003).

However, the taxa that have been found distinctive and predominating in sediment sample 6 were absent from the brackish sediments. The predominance of freshwater taxa was also different from those in brackish sediments. Several studies have shown that the microbial community composition in the freshwater is different from those in saline environments (Bouvier and del Giorgio, 2002; Henriques *et al*., 2006; Jiang *et al*., 2007). Nevertheless, Wu *et al*. (2006) indicated that salinity could result in small overlaps of freshwater and saline bacterial community compositions and they suggested that there are two possible strategies that can occur in a closed system by which a microbial community can respond to increased salinity; i) species replacement and ii) gradual adaptation of the same taxa. Furthermore, Hobbie (1988) also stated that bacteria in fresh-salt waters systems generally have different physiological means in tolerating the given level of salt concentrations. Taxa that can tolerate water salinity changes whilst maintaining the same metabolic capabilities will therefore be selected (Hart *et al*., 1991; Nielsen *et al*., 2003). The distinctiveness of sediment sample 6 is likely to suggest that the incursion of seawater has resulted in the mixing with the

freshwater lake of Suigetsu during the deposition of sediment 6 at 82.66 cm (351 BP) which has ultimately resulted in the diversification of bacterial taxa deposited in sediment sample 6. Additionally, species that is intolerant to increasing salinity will be replaced by species that can better tolerate to the changes (Hart *et al*., 1991; Nielsen *et al*., 2003; Wu *et al*. 2006), therefore the observation of different bacterial taxa in the brackish sediments compared to the freshwater.

The mixing sediment sample 6 that contained unique and distinctive taxa from the rest is highly likely the result from the seawater invasion during 1664 AD. These taxa are uncommon and most of them are found in marine habitats. The major taxa include *Oxalobacteraceae*, *Comamonadaceae*, *Sphingomonadaceae*, *Rhodobacteraceae*, *Caulobacteraceae*, *Moraxellaceae* and *Flavobacteriaceae*. *Oxalobacteraceae* belongs to *Betaproteobacteria,* and has been detected in freshwater sediment of Lake Washington (Nercessian *et al*., 2005) and it was found particularly predominant in Lake Michigan, a freshwater habitat in the coastal zone (Mueller-Spitz *et al*., 2009). *Comamonadaceae* also belongs to the beta subclass of *Proteobacteria* was also isolated from freshwater environments in Lake Kinneret, Israel. It has been characterised as Gram-negative bacteria and is a strict aerobe (Gomila *et al*, 2010). In the lake sediment of Lake Washington, *Comamonadaceae* was detected too (Nercessian *et al*., 2005). However information on these taxa in lake waters system is limited.

In addition, *Sphingomonadaceae* with oligotrophic characteristics are widely found in soils, corals, eutrophic waters, plant surfaces and drinking water (Murakami *et al*., 2010; Reddy *et al*., 2007; Thomassin-Lacroix *et al*., 2001; Vaz-Moreira *et al*., 2011). In particular *Sphingomonad* is a genus that has been a focus of studies on their ability to degrade xenobiotic compounds, aromatic hydrocarbons and environmentally hazardous compounds (Aso *et al*., 2006; Cavicchioli *et al*., 1999; Matsuyama *et al*., 2007; Stolz, 2009). Besides, due to their oligotrophic character, they were also found to be able to survive in chlorinated waters by forming biofilms (Hong *et al*., 2010). *Sphingomonads* are said to be ubiquitous in aquatic environments (Vaz-Moreira *et al*., 2011) and strains of this genus that were detected in marine environments are not known about their roles in marine environments (Cavicchioli *et al*., 1999; Murakami *et al*., 2010).

Similarly, *Rhodobacteraceae* can be found abundantly in saline environments (Kompantseva *et al*., 2010; Shigematsu *et al*., 2009). *Rhodobacteraceae* is a family in the alpha subclass of *Proteobacteria* (Shigematsu *et al*., 2009). Previous studies found that haloalkaliphilic purple non-sulphur bacteria belonging to this family are widely distributed in shallow soda lakes in Central Asia (Kompantseva *et al*., 2005; 2007; 2009; 2010). These bacteria are found to be able to grow anaerobically in the presence of light (photoheterotrophically) as well as aerobically in the dark (chemoheterotrophically) (Kompantseva *et al*., 2010). *Roseobacter* is a genus of *Rhodobacteraceae* that is a major group found in diverse marine environments, including coastal, open oceans, sea ice and coastal sub seafloor sediment (Brinkmeyer *et al*., 2003; González and Moran, 1997; Inagaki *et al*., 2003; Selje *et al*., 2004).

Caulobacteraceae is a family in alpha subclass of the phylum *Proteobacteria*. The genera that belong to *Caulobacteraceae* family such as *Caulobacter* can be detected in both freshwater and marine environments while *Roseobacter* and *Brevundimonas* are mainly found in the marine habitats (Buchan *et al*., 2005; Hagström *et al*., 2000; Stahl *et al*., 1992). However, understanding of this family in aquatic ecology is limited. Likewise, the *Moraxellaceae*, which belongs to the gamma subclass of the phylum *Proteobacteria*.

Flavobacteriaceae belongs to the *Bacteroidetes* (Niu *et al*., 2011). The genera of *Flavobacteriaceae* that have been described can be isolated mainly from marine-like habitats (Bowman *et al*., 1997, 1998; Gosink *et al*., 1998; Bowman and Nichols, 2002). Recently, members of the family *Flavobacteriaceae* were detected in a shallow freshwater eutrophic lake of Lake Taihu. This study showed that the members of the family *Flavobacteriaceae* are the major *Bacteroidetes* which predominate during the blooming of diatoms (Niu *et al*., 2011). Likewise, *Flavobacteriaceae* that have been detected abundantly in seawater ecosystems have also been shown to respond to algal blooms (Pinhassi *et al*., 2004).

Last but not least, in order to assess the similarity of bacterial communities between the water column and lake sediments of Lake Suigetsu, a study by Kondo *et al*. (2009) was used as a reference. Taxa that have been detected in the anoxic bottom water included sulphate-reducing bacteria, *Bacteroidetes*, *Gammaproteobacteria* and green sulphur bacteria. *Bacteroidetes* on the other hand were detected throughout the water

column. Ideally, these taxa should correlate to those detected in the brackish sediments as portrayed in the PLS-DA analysis, however this is not the case. In general, *Firmicutes* are the major components found in both freshwater and brackish sediments. Previous studies of Lake Suigetsu by Kondo and Butani (2007) also found that the diversity and abundance of the SRB population between these environments was significantly different. Swan *et al*. (2010) explained that particle-associated bacterial communities in sediment are fundamentally different to the free-living microbial populations in the water column. Biderre-Petit *et al*. (2011) also stated that microbial activities in a lake sediment are much greater than in the lake waters, which may also explain the dissimilarity of bacterial community composition in these habitats. Moreover, nutrient status in the sediments could serve as a more important factor rather than salinity, which therefore may also explain this occurrence.

5.5. Conclusions:

In summary, based on metagenomics analysis, the event of seawater incursion in Lake Suigetsu is likely to occur between 349 BP and 352 BP (82.16-83.16 cm) which fall within sediment sample 6. The incursion of seawater may have caused the mixing of undisturbed lake waters of Lake Suigetsu, resulting in a bloom of bacteria that was not the characteristic of the stratified lake we see today. Metagenomics analysis has enhanced the species information of DGGE data to a higher level which has enabled the differentiation of bacterial community structure and composition from different water conditions. This study also suggests that replacement of species and cell inactivation is likely to occur when the chemical condition of Lake Suigetsu changes from freshwater to brackish. According to Hobbie (1988) slow evolution of bacterial taxa may occur especially in larger lake ecosystems, when taxa originally adapted to saline conditions need to adapt to a change of water conditions to freshwater. The brief comparison of lake water taxa to lake sediment's suggests that these environments are fundamentally different and therefore there is a different bacterial composition.

Chapter 6 Analysis of the bacterial community in ancient sediments deposited during a transition from a cooler to warmer climatic conditions

6.1. Introduction

Lakes are sensitive to environmental variations (Battarbee, 2000), in particular, freshwater ecosystems, are vulnerable to climatic changes (Lake *et al*., 2000; Woodward *et al*., 2010). The changes in climatic conditions are positively correlated to the variations of lakes' water temperatures and due to the restriction of dispersal capabilities, the species within these habitats receive direct impacts from adjacent terrestrial ecosystems as well as through numerous anthropogenic activities such as pollution (Hornung and Reynolds, 1995; Lake *et al*., 2000; Woodward *et al*., 2009). Therefore, aquatic lake ecosystems can detect early signals of wider scale change for which they are also known as the 'sentinel systems' (Woodward *et al*., 2010).

Lake systems have high sedimentation rates and the movement of organic matter, nutrients, pollutants and continuous accumulation of sediments will influence microbial community structure and function in the lake sediments (Battarbee, 2000; Lake *et al*., 2000). Previous studies have addressed the effects of climate change on the terrestrial and aquatic habitats (Heino *et al*., 2009; Heywood and Watson 1995) and these studies were mainly focused on individuals or species populations rather than the higher levels of organisation (i.e. communities). Moreover, the study of the effects of climate change on aquatic sediments is also limited (Lake *et al*., 2000) and comprehensive understanding of climate change and predictive capability of its effects on biodiversity in various organism groups and ecosystems are still lacking (Heino *et al*., 2009). In fact, the biota in sediment and lake waters are diverse and abundant, in which they have resilient influence on the ecosystem processes including the primary production and nutrient dynamics (Palmer *et al*. 1997). The changes of atmospheric and climatic conditions are said to have impact on both abiotic and biotic drivers in the ecosystems (Castro *et al*., 2010). In the water column and lake sediments, bacteria not only act as decomposers of inorganic and organic materials (Felip *et al*., 1996; Gurung and Urabe, 1999), but also primary producers of certain organic carbons through the consumption and assimilation of dissolved organic carbon (DOC) (White *et al*., 1991). Besides

nutrients availabilities, temperature is often suggested to be another primary driver responsible for the regulation of bacterial growth rates (Gurung and Urabe, 1999). In many studies, temperature has shown to have affected bacterial growth rates (Castro *et al*., 2010; Shiah and Ducklow, 1994; White *et al*., 1991), bacterial productions (Shiah and Ducklow, 1994), abundance (Shiah and Ducklow, 1994), compositions and diversity (Linström *et al*., 2005).

In this study, the bacterial community in the sediment records from past climate, during the transition from colder to warmer climate will be assessed. The annually laminated sediments of Lake Suigetsu have recorded the events of past climatic changes and provide precise chronology for past-climate reconstruction (Nakagawa *et al*., 2002, Kossler *et al*., 2011). High resolution pollen analysis based on the SG4 sediment core (recovered in 1993) by Yasuda *et al*. (2004) has demonstrated that there were significant changes in vegetation around Lake Suigetsu from 15, 000 to 14, 500 BP based on Suigetsu's varve years. Subalpine coniferous and cool-temperate trees (*Pinus* subgen. *Haploxylon*, *Picea*, *Tsuga* and *Betula*) drastically decreased after 15, 000 BP and following 14, 500 BP, a constant increase in temperate trees (*Fagus crenata*, *Quercus* subgen. *Lepidobalanopsis*, *Carpinus* and *Cryptomeria japonica*) was detected. This period from 15, 000 to 14, 500 BP marked the transition of ecology from glacial to postglacial ecosystems which also corresponds to the Bølling interstadial that occurred in the Europe (Kitagawa and van der Plicht, 1998b; Nakagawa *et al*., 2005; Yasuda *et al*., 2004). However, when comparing to the radiocarbon dating from the North Atlantic region, there was a difference by a few centuries earlier (Nakagawa *et al*., 2003). The ecological transition was completely established by 14, 500 BP. The climatic conditions during this period of time were moist and warm, which would have also favoured the stable growth of temperate deciduous broad-leaved forest around Lake Suigetsu.

For studies associating with past environments, researchers usually employ fossilised organic components (i.e. lipids) as they provide an archive of ancient aquatic microbial communities that can be used to reconstruct variations in climate and their impacts on biodiversity (Brassell *et al*., 1986; Schouten *et al*., 2001). However, the analysis of these data is commonly complicated by the limited source and specificity of some biomarkers, such as lipids and pigments that are often lack of diagnostic features (Coolen *et al*., 2004; Coolen *et al*., 2008; Coolen and Gibson, 2009). According to Coolen and Overmann (2007), the analyses of fossil 16S rRNA gene sequences in

subsurface sediments not only can provide independent evidence for the occurrence of microbial community but also allow a more detailed reconstruction of their paleoenvironment.

Therefore, fossil DNA will be utilised in this study instead, as fossil DNA is well-preserved in meromictic sediment records, as demonstrated in Chapter 4. Former studies of Lake Suigetsu have not examined this area of study, hence, we aim to explore the effects of climate change on ancient bacterial diversity and its community structures, to determine if specific taxa had strong correlation to different climatic conditions and if bacterial taxa demonstrate a measurable change during the transition from glacial climate to postglacial, dating from 15, 000 to 14, 500 BP. This period of time is targeted as the abrupt warming of earth around this epoch was more significant than the transition from the Younger Dryas to Pre-boreal (11, 600–11, 400 BP) (Yasuda *et al*., 2004).

6.2. Experimental strategy:

In order to study the relationship between climate changes and ancient bacterial communities, sediment records with information of climate transition from glacial to postglacial were targeted from 14,983 BP to 14, 400 BP, at 1740.70 to 1696.10 cm. Fossil DNA extraction was carried out for a total of 46 sediment samples, followed by PCR-DGGE of bacterial 16S rDNA analysis. Based on pollen records, there were eight sediment samples taken from warmer region, 32 sediment samples from the transition zone while six sediment samples from the colder region. Besides, ten out of the 46 sediment samples were selected for metagenomics sequencing to further identify the composition of ancient bacterial community from different climate conditions.

6.3. Results:

6.3.1. Sediment sampling

Samples of the sediments across the transition from cooler to warmer environmental condition, as determined from the high resolution pollen analyses (Nakagawa *et al*., 2005), were collected. These include the six samples of the cooler period, 32 samples across the transitional zone while eight samples from the sediments with vegetation profile adapted to higher environmental temperatures (Table 6.1).

Table 6.1: Characterisation of bacterial community profiles of Suigetsu core with increasing depth

Condition	Core SG06-A09	Age (BP)	Composite depth (cm)	Bacterial DGGE Profile	
				Rr (Species richness)	*H*' (Shannon-Wiener Index)
Warm	56	14400	1696.10	12	1.690
	57	14411	1697.12	10	1.714
	58	14421	1698.15	22	2.794
	59	14434	1699.17	13	2.310
	60	14445	1700.19	18	2.372
	61	14458	1701.22	16	2.406
	62	14469	1702.24	17	2.127
	63	14481	1703.26	15	1.999
			Mean	15.38	2.18
Transition	64	14493	1704.28	17	2.311
	65	14504	1705.31	17	2.265
	66	14516	1706.33	15	2.084
	67	14531	1707.35	11	1.175
	68	14544	1708.38	14	2.249
	69	14558	1709.40	11	2.145
	70	14572	1710.41	10	2.013
	71	14588	1711.43	12	2.086
	72	14604	1712.44	10	1.923
	73	14618	1713.46	10	2.026
	74	14630	1714.47	11	1.992
	75	14642	1715.49	15	2.204
	76	14653	1716.50	16	2.381
	77	14664	1717.47	20	2.431
	78	14679	1718.43	16	2.174
	79	14695	1719.40	11	1.966
	80	14707	1720.50	13	2.216
	81	14719	1721.40	18	2.536
	82	14728	1722.30	8	1.784
	83	14740	1723.20	11	2.098
	84	14751	1724.10	11	1.634
	85	14762	1725.00	14	2.133
	86	14775	1725.90	11	1.832
	87	14786	1726.80	17	1.630
	88	14799	1727.70	12	1.443
	89	14809	1728.60	14	2.415
	90	14824	1729.63	9	1.362
	91	14837	1730.67	18	1.880
	92	14852	1731.70	13	1.536
	93	14865	1732.70	13	1.506
	94	14881	1733.70	11	1.481
	95	14895	1734.70	11	1.463
			Mean	12.5	1.95
Cold	96	14910	1735.70	17	1.741
	97	14925	1736.70	14	1.597
	98	14939	1737.70	9	1.245
	99	14952	1738.70	11	1.652
	100	14967	1739.70	13	1.959
	101	14983	1740.70	10	1.765

Composite depth is taken from the midpoint of each of the 1 cm^3 sediment sample. Each sediment age was measured using Level Finder Software 4.3.3. Each sediment age represents the age at the midpoint of the composite depth. Rr = species richness. Rr was calculated from each DGGE lane. *H*' = Shannon Weiner Index of diversity. *H*' was calculated using ln *x* (natural logarithm) of the normalised DGGE data; i.e. ln (*x*)***x*.

6.3.2. Reproducibility of the bacterial community DGGE Data

To examine the reproducibility of the result, two sediment samples from the upper cores (SG06-A09 60 and 67) and two from the deepest depths (SG06-A09 90 and 97) were chosen randomly. Selected sediment samples were extracted in triplicate and analysed using PCR-DGGE. Figure 6.1 showed that the bacterial DGGE profile of each sediment sample in triplicate are effectively identical to one another. This indicates that the bacterial profiles are consistent and reproducible with no significant difference in the number of taxa identified.

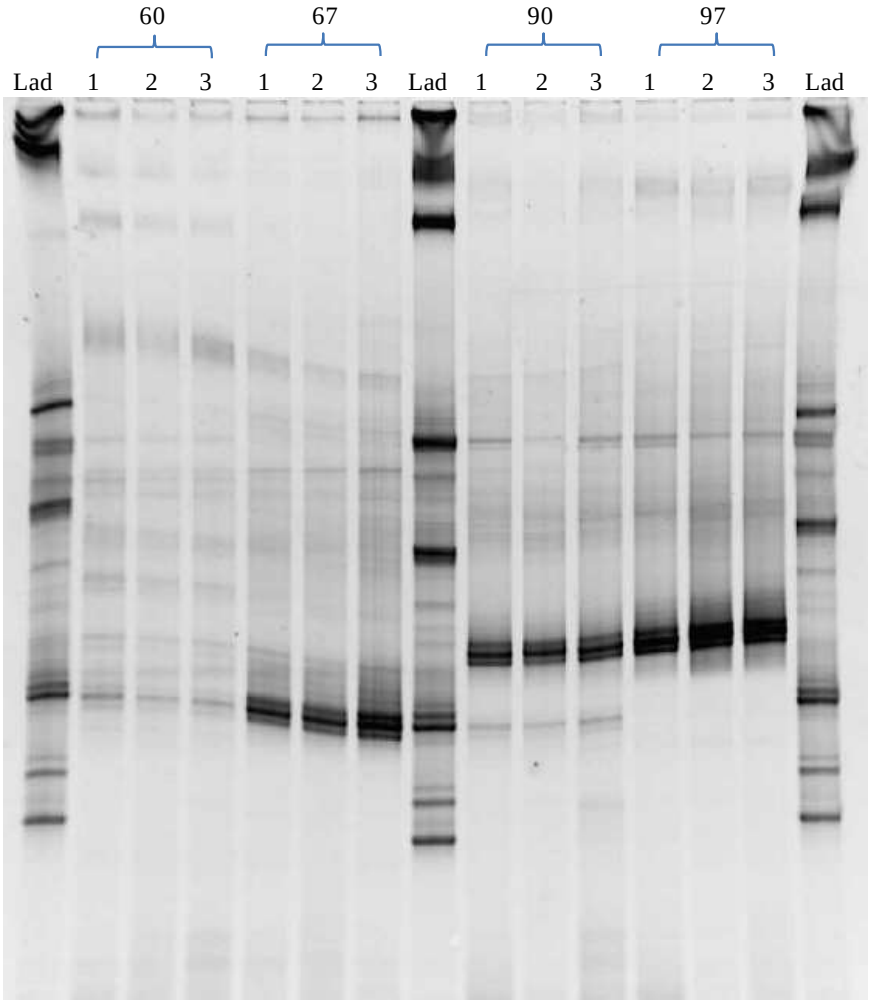


Figure 6.1: Bacterial DGGE profile of sediment sample SG06-A09 60, 67, 90 and 97 in triplicate. Figure shows bacterial 16S rDNA genes based on DNA analysis. Composite depth of A09-60 = 1700.19 cm (14,445 BP), 67 = 1707.35 cm (14,531 BP), 90 = 1729.63 cm (14,824 BP) and 97 = 1736.70 cm (14,925 BP). Lad = ladder.

6.3.3. DGGE Analysis of Bacterial Community before and after Climate Changed

Bacterial DGGE fingerprints profiles from sediments deposited in cooler to warmer climatic conditions were produced (Figure 6.2a and 6.2b). SG06-A09 56 to 63 represents sediment samples from warmer climate while sediment samples SG06-A09 96 to 101 falls in the colder climate zone. In warmer climate sediments, both the number of taxa (mean = 15.38) and bacterial diversity (mean = 2.18) measured were higher than those in the sediments from the cooler climatic condition, which have average values of 12.33 and 1.66, respectively (Table 6.1).

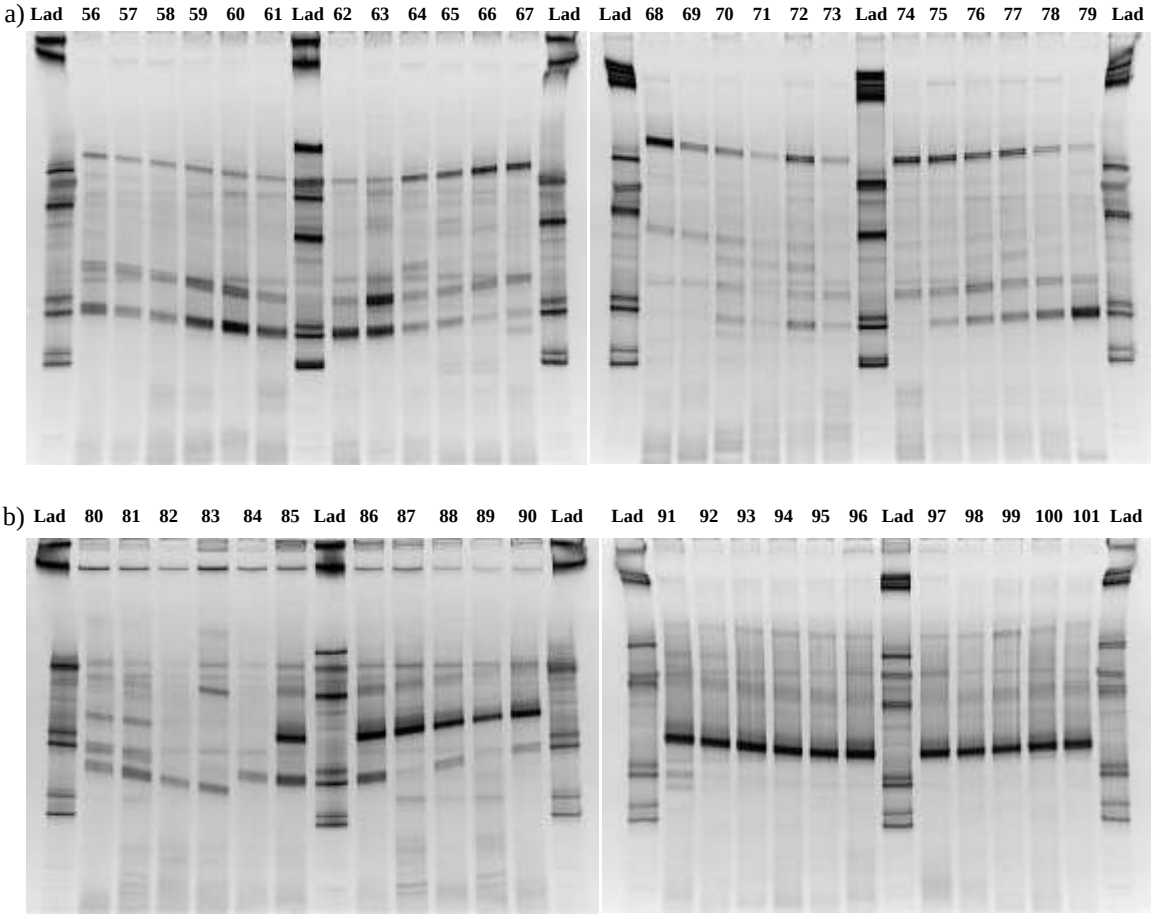
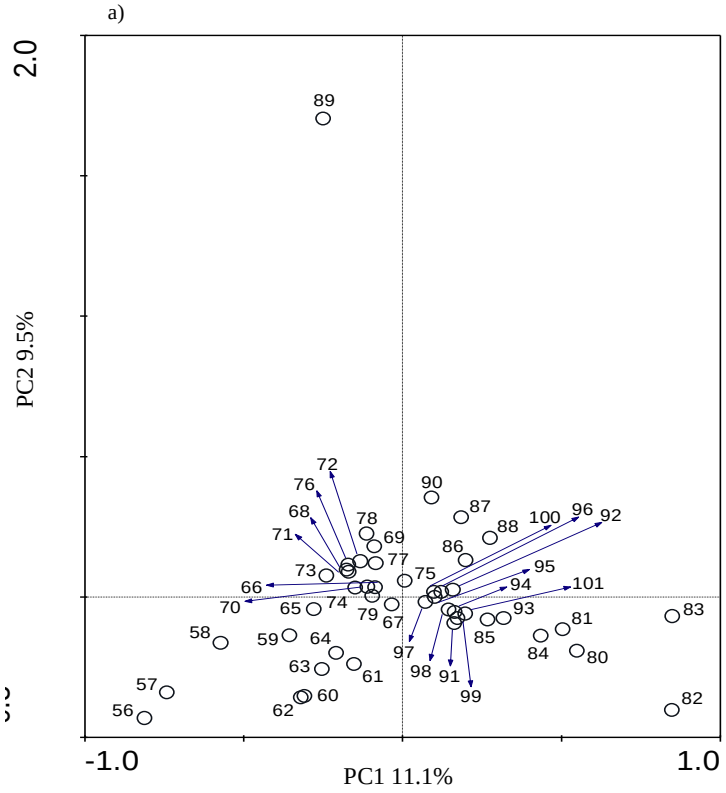


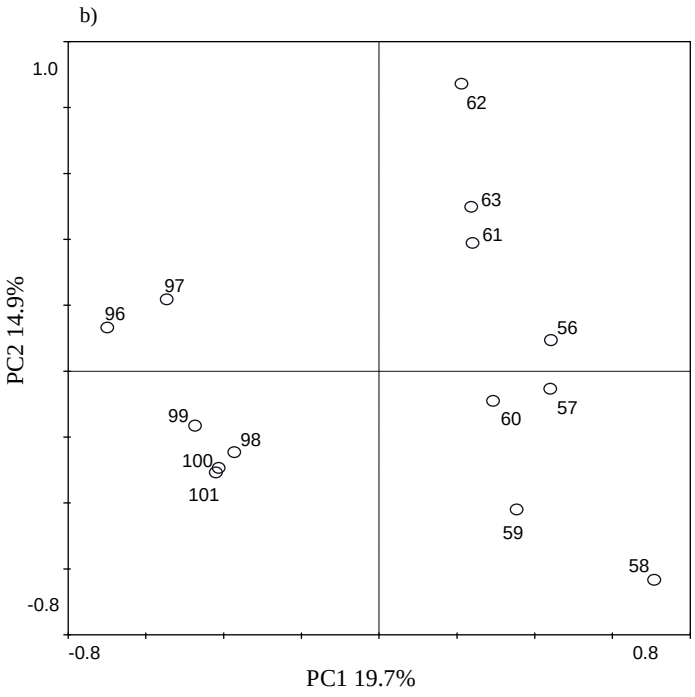
Figure 6.2: DGGE profile of bacterial 16S rDNA genes based on DNA analysis. a) sediment samples SG06-A09, 56-63 fall in warm climate zone from depth 1696.10 cm to 1703.26 cm (14400 BP to 14481 BP), sediment samples 64-79 are in the transition zone at sediment depth 1704.28 cm to 1719.40 cm, (14493 BP to 14695 BP); b) sediment samples 80-95 are in the transition zone from depth 1720.50 cm to 1734.70 cm (14707 BP to 14895 BP), sediment samples 96-101 are in cold climate zone at depth 1730.67 cm to 1734.70 cm (14837 BP to 14895 BP).

6.3.4. PCA analysis

The initial PCA analysis was carried out to compare the bacterial community structures between all the sediment samples. Both PC1 and PC2 accounted for only 20.6% of the total variance, and this suggests that the difference between the communities was relatively low (Figure 6.3a). There was some indication which was similar to the outcome observed when sediment samples from the transition zone were excluded. Both the principal components which accounted for a total variance of 34.6% indicates a weak support of climatic effect on bacterial communities (Figure 6.3b).

Besides, when bacterial communities were analysed within a climatic condition, principal components showed a greater variance. In warmer climate, sediment samples SG06-A09 58, 62 and 63 exhibited great correlations to PC1 axis which have accounted for 26% of the total variance in the community while sediment samples of SG06-A09 56, 57 and 60 appeared to have correlations to PC2 axis that explained 18.6% of the variance (Figure 6.3c). Likewise, in cold climatic condition (Figure 6.3d), SG06-A09 96, 97, 98 and 101 were closely distributed along the PC1 axis, indicating a great correlation to PC1 axis as explained by 44.2% of the total variance in the communities. Sediment samples SG06-A09 99 and 100 on the opposite showed correlations to PC2 axis which accounted for 23.8% of the remaining variance. Conversely, bacterial communities in transition zone showed weak correlations to both the principal components which accounted for only 25.1% of the total variance in the communities (Figure 6.3e).


a)
2.0
PC2 9.5%
89
72
76
68
71
73
66
70
58
57
56
90
87
88
100
96
92
78
69
86
77
75
95
94
101
65
74
79
67
93
81
83
59
64
63
61
60
62
97
98
91
99
85
84
80
82
-1.0
1.0
PC1 11.1%


b)
1.0
PC2 14.9%
62
63
61
97
96
56
57
60
99
98
100
101
59
58
-0.8
-0.8
0.8
PC1 19.7%

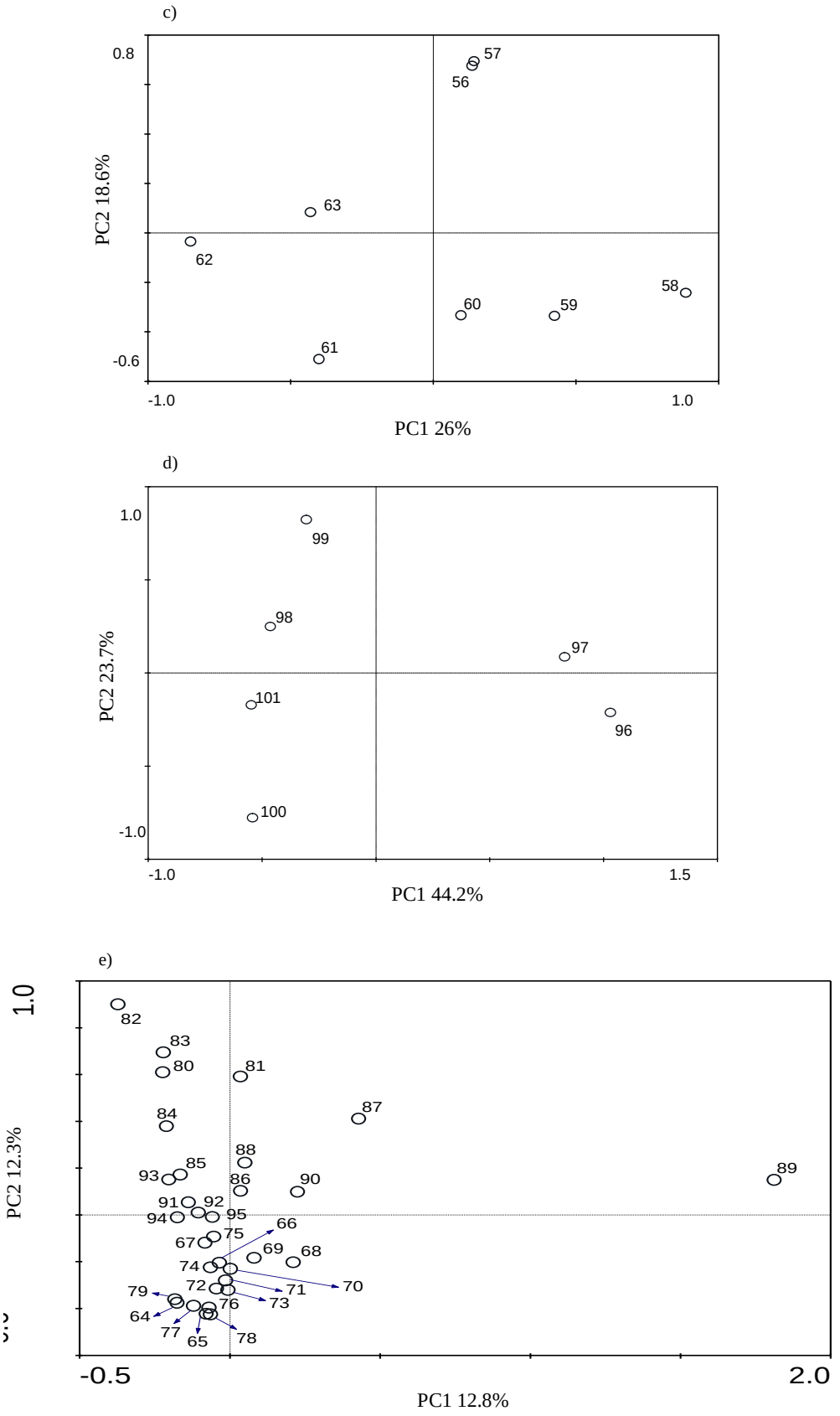


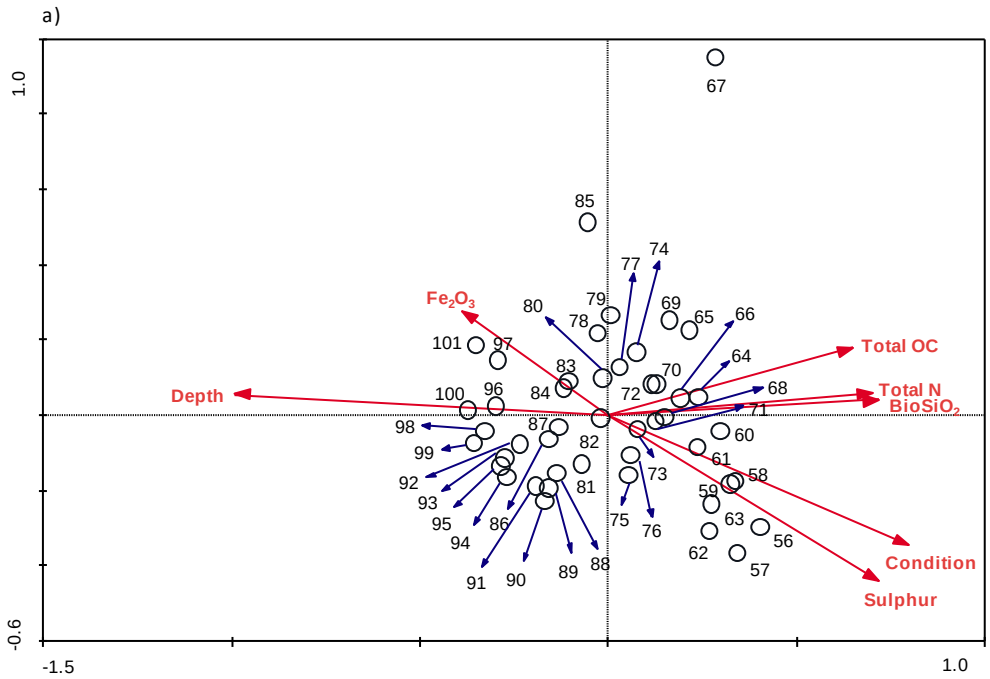
Figure 6.3: PCA analysis of bacterial communities under different climate conditions. a) Bacterial communities across climatic changes from cold to warm; b) bacterial communities during cold and warmer climates; c) bacterial communities in warmer climate; d) bacterial communities in cold climate; e) bacterial communities within transition zone. Sediment sample 56 to 63 = warmer climate, sediment sample 64 to 95 = transitional zone, sediment sample 96 to 101 = colder climate.

6.3.5. Raup-Crick Similarity index

Following PCA analysis, a pairwise comparison of each sample was performed using the Raup and Crick Similarity Index analysis (Appendix 11). This analysis was used to test if similarities observed between sediment profile patterns were significantly higher or lower than would be observed by chance. This analysis showed that in the sediment, adjacent samples were frequently significantly similar to the community in the sample immediately above and below but rarely so with communities from sediment layers further away.

6.3.6. Multivariate analysis of microbial communities' structure

RDA analysis was subsequently carried out to evaluate the relationship between community structures and environmental variables including sediment depth, percentage organic carbon, nitrogen and sulphur determined from analysis of sediment samples and Iron (III) oxide and biogenic silica oxide data previously available (Nakagawa *et al*., 2005). Bacterial communities' structures appeared to be significantly affected by sediment depth (p = 0.002) and sulphur contents (p = 0.038). Total organic carbon (p = 0.058) also appear to be a driver for community structure (Figure 6.4a; Table 6.2). Analysing all samples simultaneously can result in outliers constraining the analyses. Therefore, we analysed bacterial profiles from warm, transitional and cooler sediment in isolation to ameliorate this effect. This showed in both warmer and cooler sediment profiles, the structures of bacterial communities were significantly affected by sediment depth (p = 0.024 and 0.032, respectively), (Figure 6.4b and c; Table 6.2). During the transitional period, total organic carbon (p = 0.05) and biogenic silica oxide (p = 0.002) appeared to have significantly impacted on the structures of bacterial populations. In addition, while not significant, sediment depth and total nitrogen also seemed to be drivers of the bacterial community structures (p = 0.066 and 0.06, respectively) (Figure 6.4d; Table 6.2).


a)
1.0
-0.6
-1.5
1.0
Depth
Fe2O3
Total OC
Total N
BioSiO2
Condition
Sulphur
67
85
74
77
80
79
78
69
65
66
101
97
83
64
70
72
68
100
96
84
71
98
87
82
60
99
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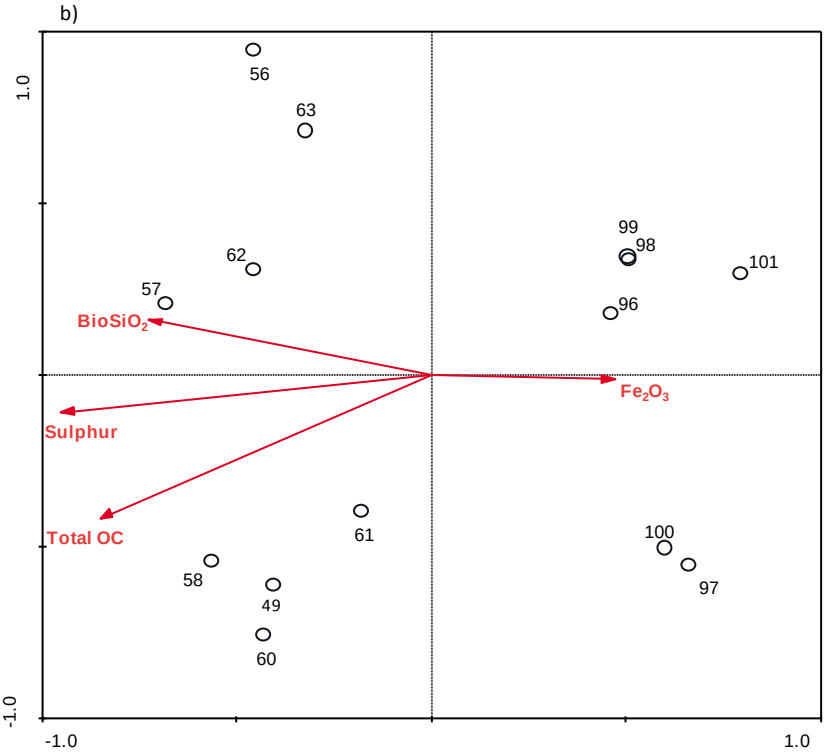
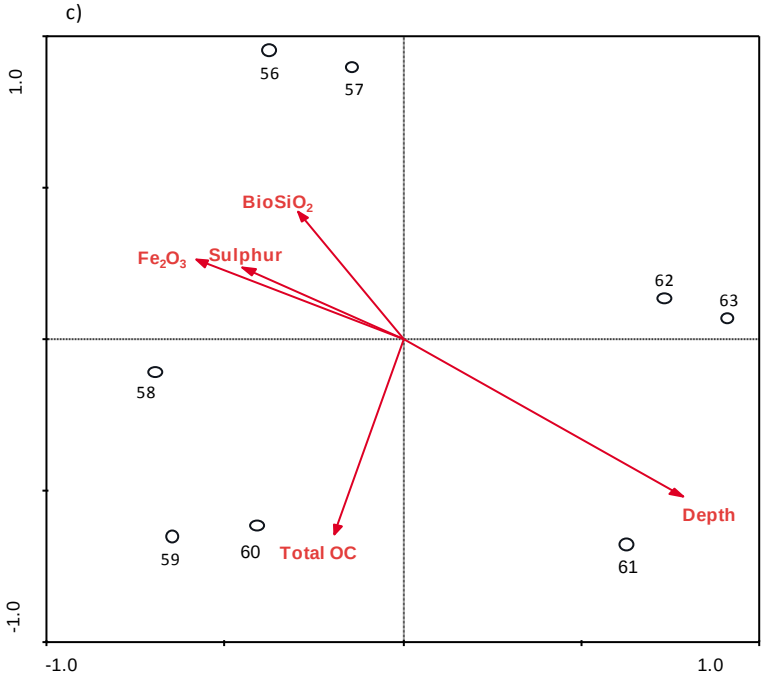
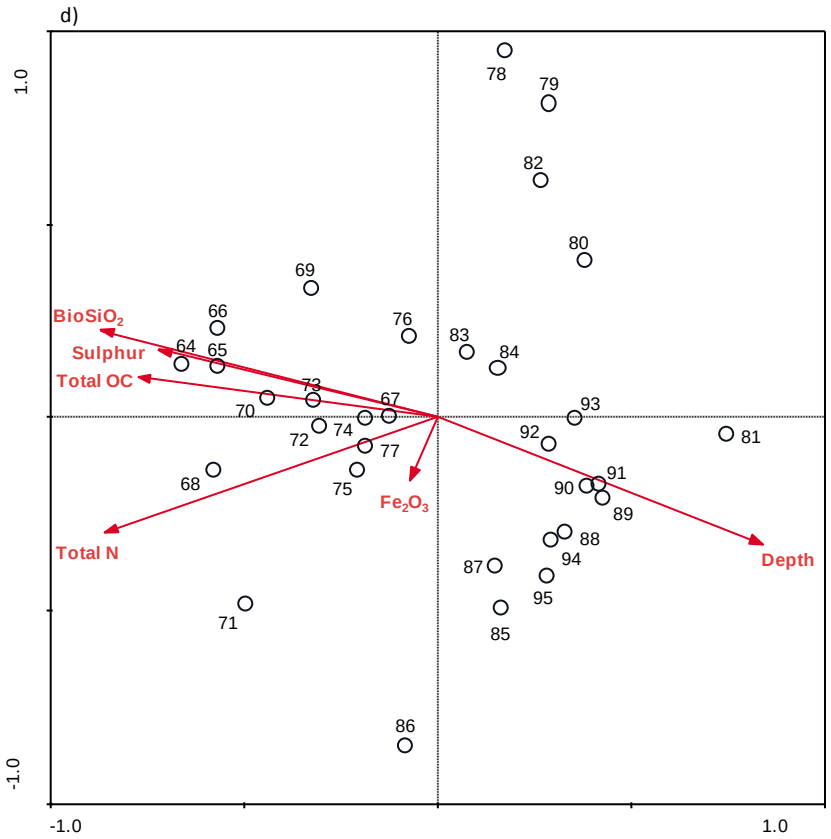
76
63
94
56
62
88
91
90
89
57


b)
1.0
-1.0
-1.0
1.0
56
63
62
99
98
101
57
96
BioSiO2
Fe2O3
Sulphur
Total OC
61
100
58
97
49
60


c)
1.0
-1.0
-1.0
1.0
56
57
BioSiO2
Fe2O3
Sulphur
62
63
58
59
60
Total OC
61
Depth


d)
1.0
-1.0
-1.0
1.0
78
79
82
80
69
BioSiO2
66
76
83
84
64
65
Sulphur
Total OC
73
67
70
93
74
72
92
81
77
68
75
Fe2O3
91
90
89
88
Total N
94
Depth
87
95
71
85
86

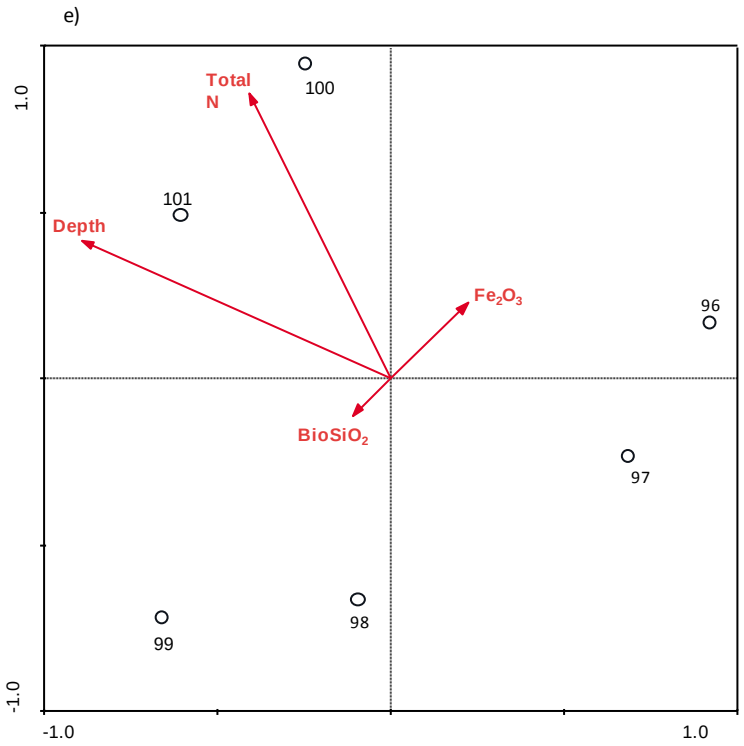


Figure 6.4: RDA analysis of bacterial communities' structures under varying climatic conditions. a) Bacterial communities' structures from colder to warmer climate; b) bacterial communities' structures during cold and warm climates; c) bacterial communities' structures during warmer climate condition; d) bacterial communities' structures during transition period; e) bacterial communities' structures during colder climate condition. Sediment sample 56 to 63 = warmer climate, 64 to 95 = transition zone, 96 to 101 = colder climate. Depth = sediment composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3 = Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$ = biogenic silica oxide, Condition = different climatic conditions

Table 6.2: Significant evaluation of environmental factors against bacterial community structures based on RDA Analysis

	Conditional Effects				
	Cold to Warm	**Cold/Warm**	**Warm**	**Transition**	**Cold**
Variable	*p*	*p*	*p*	*p*	*p*
Depth	0.002	-	0.024	0.066*	0.032
Total OC	0.058*	0.250	0.424	0.050	-
Total N	0.106	-	-	0.060*	0.284
Fe_2O_3	0.756	0.206	0.380	0.820	0.260
Sulphur	0.038	0.002	0.660	0.642	-
$BioSiO_2$	0.764	0.372	0.436	0.002	0.402
Condition	0.332	-	-	-	-

Highlighted *p* in red represents significance value. $p < 0.05$ is considered to have significant correlation between bacterial community structures and environmental factors while $p > 0.05$ would be less significant. Depth = composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3 = Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$ = biogenic silica, Condition = different climatic conditions. Those with no input were excluded in the analysis as inflation factor (IF) is higher than 20. * indicates weak correlation of environmental factors to bacterial community structure.

6.3.7. Pearson correlation coefficient analysis of microbial diversities

Pearson correlation was also carried out to investigate environmental factors against bacterial diversity. According to this analysis, all the environmental parameters for which data was available appeared to have significantly affected the diversity of bacterial communities showing $p < 0.05$ when bacterial communities between warm and cool sediments were compared (Table 6.3). In colder sediment deposits, bacterial diversity appeared to have significant relationship with the level of total nitrogen in lake sediments and during the transition period, bacterial diversity was significantly correlated with sulphur, biogenic silica oxide, iron oxide concentrations and sediment depth. In the sediment deposited during warmer conditions, bacterial diversity appeared to have no significant correlation to any changes of the environmental parameters.

Table 6.3: Significant evaluation of environmental factors against bacterial diversity based on Pearson Correlation

Env. Variables		**Cold – Warm**	**Cold**	**Transition**	**Warm**
Total N	**Coef.**	0.410	0.760	0.261	0.556
	p	0.005	0.029	0.149	0.153
Total OC	**Coef.**	0.431	0.582	0.252	0.691
	p	0.003	0.225	0.163	0.058
Sulphur	**Coef.**	0.491	0.234	0.437	-0.085
	p	0.001	0.705	0.016	0.841
$BioSiO_2$	**Coef.**	0.505	-0.371	0.430	0.323
	p	0.000	0.469	0.016	0.479
Fe_2O_3	**Coef.**	-0.355	0.046	-0.472	0.687
	p	0.018	0.931	0.007	0.088
Depth	**Coef.**	-0.518	0.362	-0.469	0.245
	P	0.000	0.481	0.007	0.558

Table above shows relationship between bacterial diversity and environmental factors. Coef. = Pearson Correlation coefficients and *p* = p value. Highlighted *p* values ≤ 0.05 (in red) = significant correlation. A positive coefficient value = positive relationship and negative coefficient value = negative correlation. Depth = composite depth, Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3 = Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$ = biogenic silica.

6.3.8. Comparison between DGGE and Metagenomics Analysis on Bacterial Communities when Climate Condition Shifted from Cold to Warm

Sediment samples from warm (SG06 A09- 57, 58, 59), transition (SG06 A09- 67, 76, 84, 93) and cold (SG06 A09- 99, 100, 101) climate were selected for metagenomics analysis. PCA analysis of the metagenomics data showed a greater total of variance of 35.2% which was accounted for by PC1 axis. Figure 6.5a showed a clear separation of sediment samples deposited in warmer climatic conditions which were on the positive side of the x-axis from those in colder climate on the negative side of the x-axis. The DGGE data of these samples were re-analysed using PCA analysis for comparison. PCA analysis of the DGGE data (Figure 6.5b) also showed that communities from the warmer sediment were distinct from the cooler and transitional sediment communities along the principle axis accounting for 23.4% of the total variance in the communities. Both the PCA analyses based on different techniques accounted for approximately 50% of the total variance in the communities.

Multivariate analyses of CCA/RDA were again carried out for both sets of the data depending on the DCA (define this) gradient's length (< 3.5 = RDA and > 3.5 = CCA). CCA analysis was performed for DGGE data set while RDA analysis was chosen for metagenomics data. Both of these analyses showed that biogenic silica oxide has significantly affected the communities' structures of bacterial populations (p = 0.024, p = 0.034) (Figure 6.6a, 6.6b; Table 6.4).

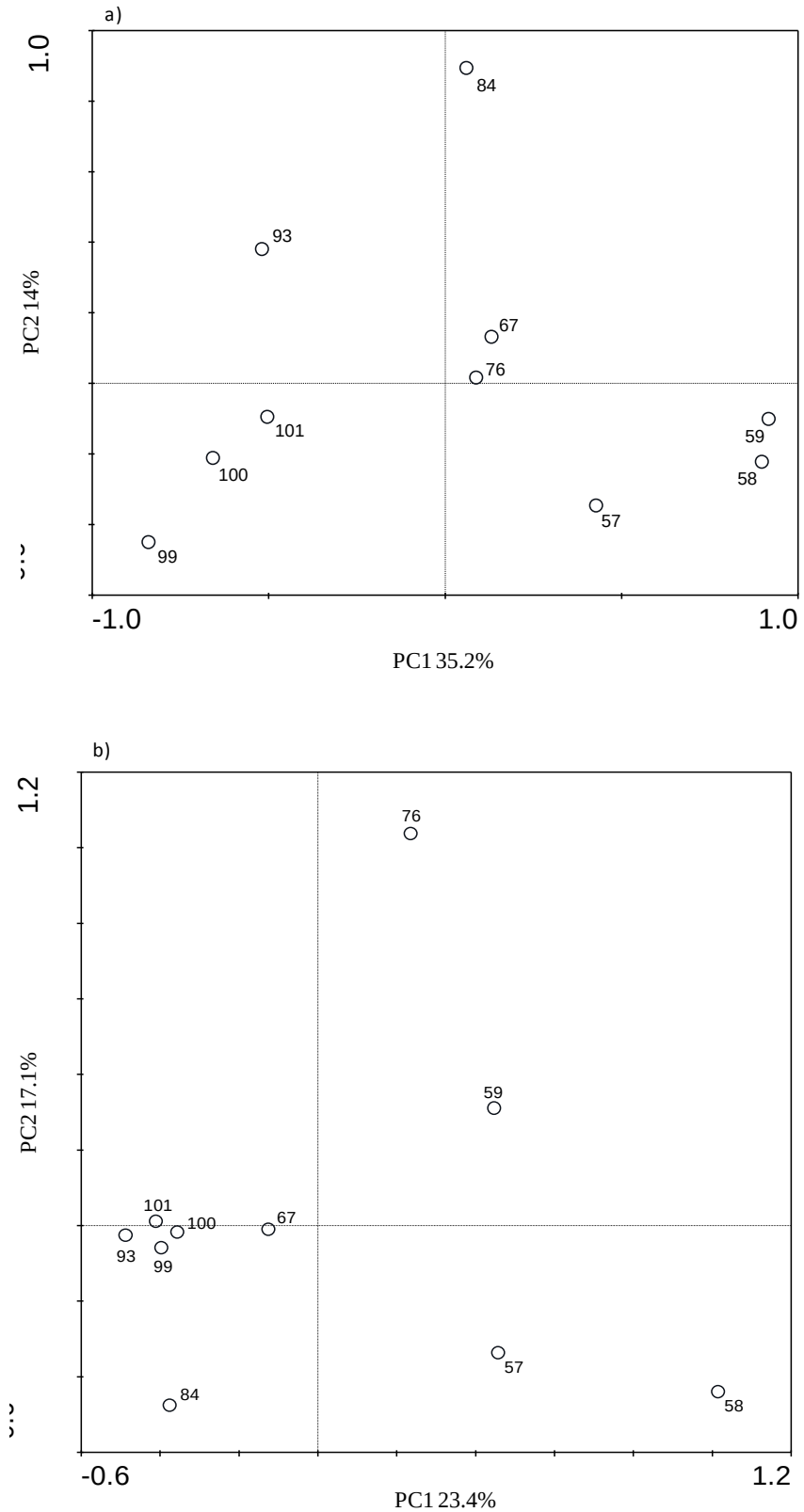


Figure 6.5: PCA analysis of bacterial communities from colder to warmer climate conditions a) Metagenomics data based on normalised OTU measurements. b) DGGE data selected for metagenomics study. Sediment samples 57-59 = warmer condition; 67, 76, 84, 93 = transition period; 99- 101 = colder condition

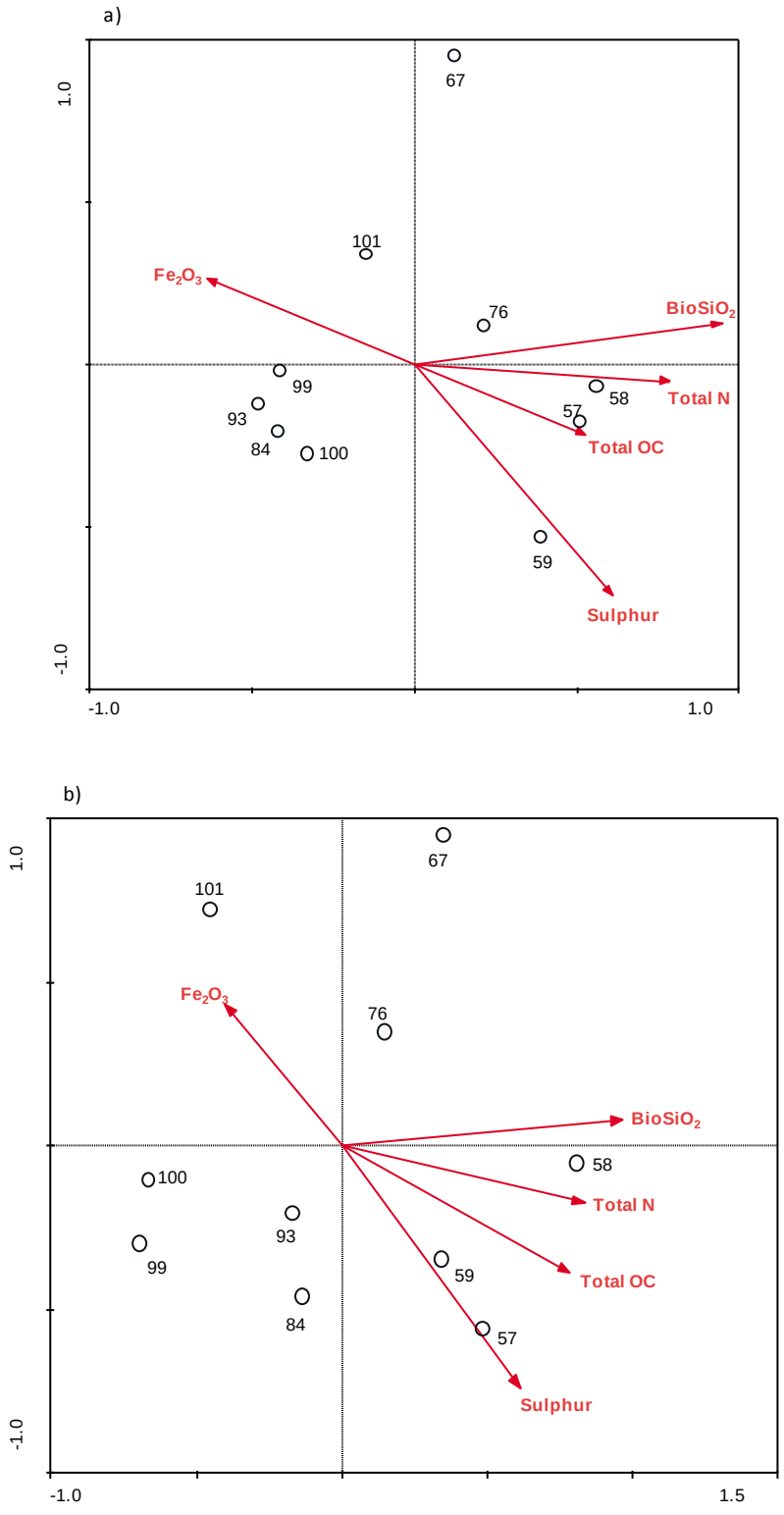


Figure 6.6: Multivariate analysis of bacterial communities. a) CCA analysis based on DGGE data. b) RDA analysis based on OTU measurements of metagenomicss data. Sediment samples 57-59 = warmer condition; 67, 76, 84, 93 = transition period; 99- 101 = colder condition

Table 6.4: Significant analysis of bacterial community structure against environmental parameters

	Conditional Effects	
	DGGE (CCA)	**Metagenomicss (RDA)**
Variable	*p*	*p*
Total OC	0.178	0.340
Fe_2O_3	0.220	0.798
Sulphur	0.090*	0.230
$BioSiO_2$	0.024	0.034
Total N	0.536	0.904

Highlighted *p* values < 0.05 (in red) = significant correlation. Total OC = total organic carbon, Total N = total nitrogen, Fe_2O_3 = Iron (III) oxide, Sulphur = sulphur content in sediment samples, $BioSiO_2$ = biogenic silica. Depth and condition were not included as the inflation factor (IF) was larger than 20. * indicates slightly significant correlation.

The community composition of the sediment communities from each sample were assembled from the metagenomics analyses. Figure 6.7 illustrates the taxa with more than 3% of the OTUs at order level present in each community profile samples. This analysis indicates that the bacterial taxa started to vary at transition sediment T84 and changes again after transition sediment T67, indicating a gradual shift in microbial taxa through the transition from sediment deposited in cooler climatic conditions to those from warmer environments. *Xanthomonadales*, *Sphingobacteriales* and *Rhodospirillales* appeared to be only detectable in colder environment while *Caulobacterales* was mainly found in warmer and transition zones. Apart from that, *Rhizobiales* and *Burkholderiales* were the major taxa that could be found in all the sediment samples.

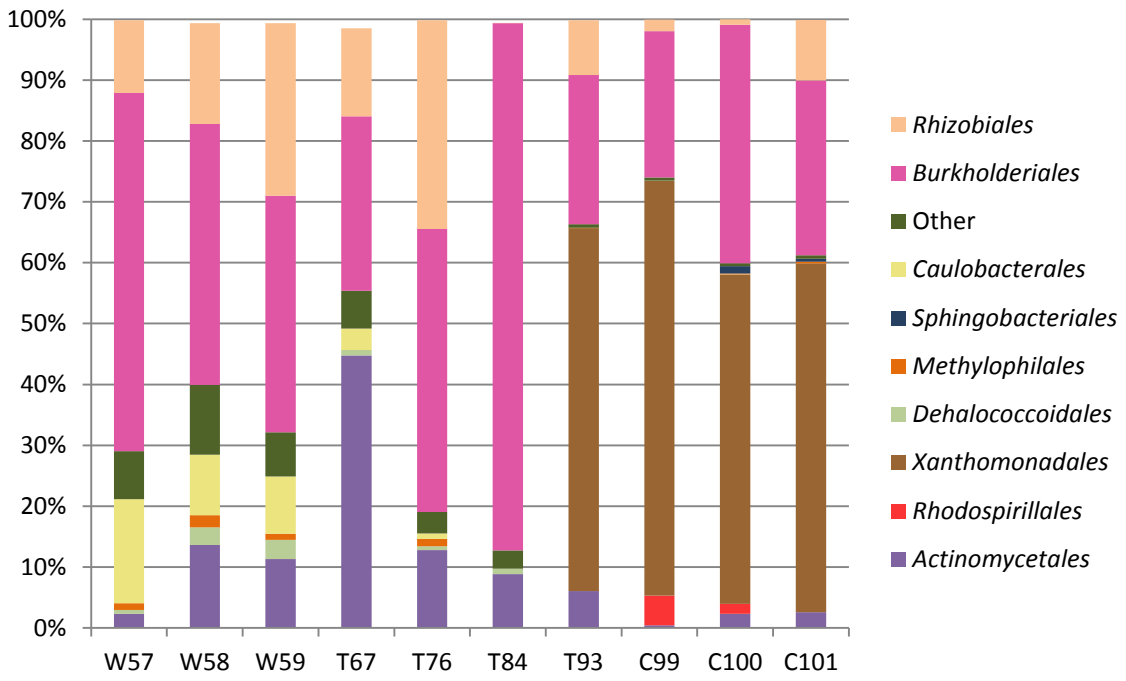


Figure 6.7: Bar chart shows taxon at order level in different environmental conditions. W = warm; T = transition, C = cold. W57 to W59 are from composite depth 1697.12 cm – 1699.17 cm (14411 BP – 14434 BP). T67 = 1707.35 cm (14531 BP), T76 = 1716.50 cm (14653 BP), T84 = 1724.10 cm (14751 BP), T93 = 1732.70 cm (14865 BP). C99 to C101 = 1738.70 cm – 1740.70 cm (14952 BP – 14983 BP). Taxa with OTU counts <3 % are grouped as 'Other'.

6.3.8.1. PLS-DA analysis of bacterial communities

To determine the relationship between taxa and the changes in measured environmental parameters principle least square discriminant analysis (PLS-DA) was used. All the ten sediment samples selected for metagenomics analysis fall inside the ellipse, indicating 95% confidence level, as defined by Hotellings T^2 *t*-test (Figure 6.8). The resulting loading plot (Figure 6.9) showed that the taxa significantly correlated with warmer sediment deposits are distinctive from both those sediments laid down during cooler or transitional climatic conditions. Moreover, the predominating taxa correlated to warmer sediment deposits were shown to be different from the prevailing taxa associated to colder sediment deposits. Taxa *Xanthomonadaceae* have demonstrated to be predominance in colder sediment deposits while *Caulobacteraceae* have shown to be prevailing in warmer sediment deposits.

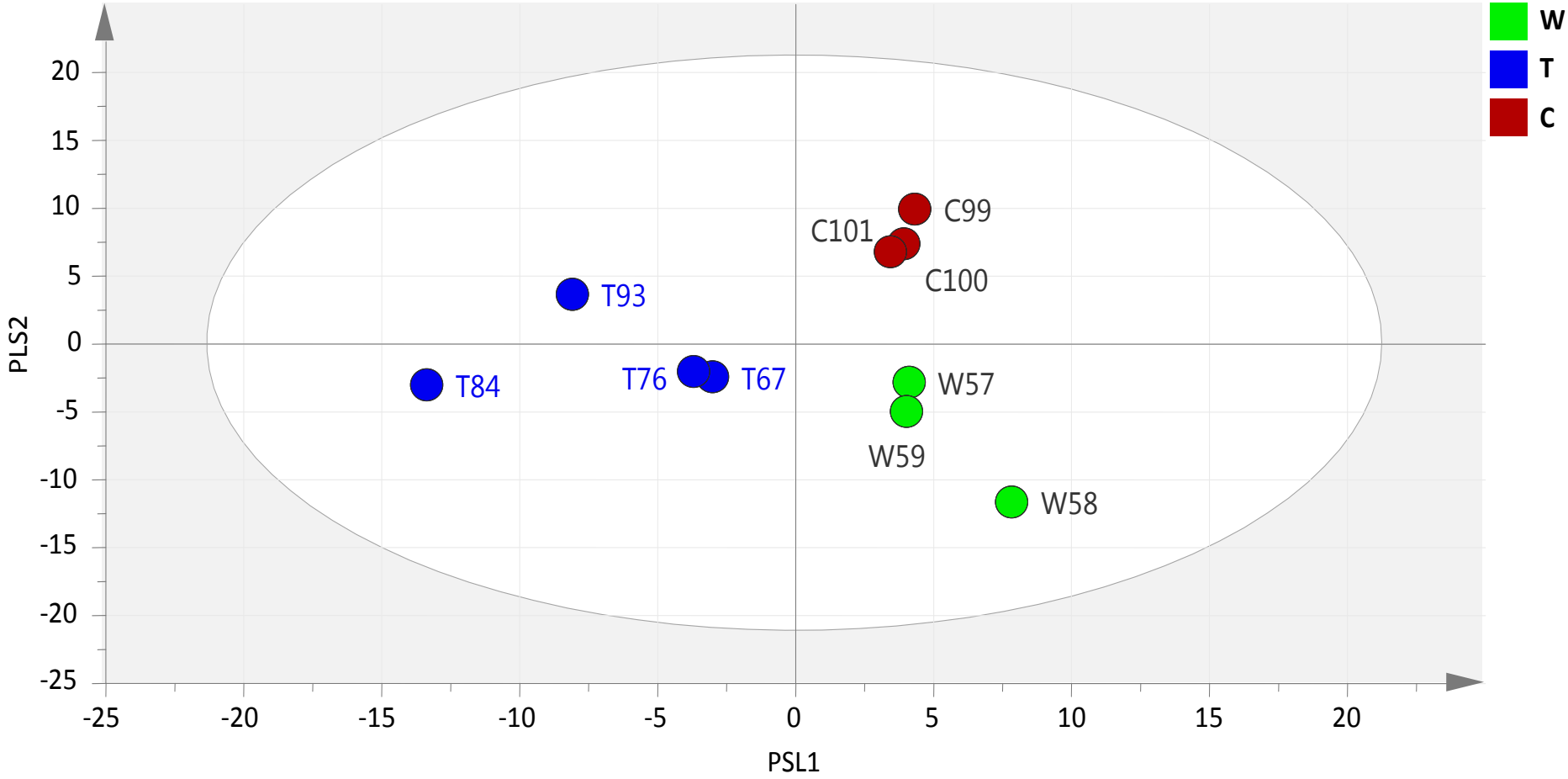


Figure 6.8: PLS-DA based on pyrosequencing data across climatic change. The ellipse denotes 95% of significance limit of the model, as defined by Hotelling's *t*-test. W = warm; T = transition and C = cold

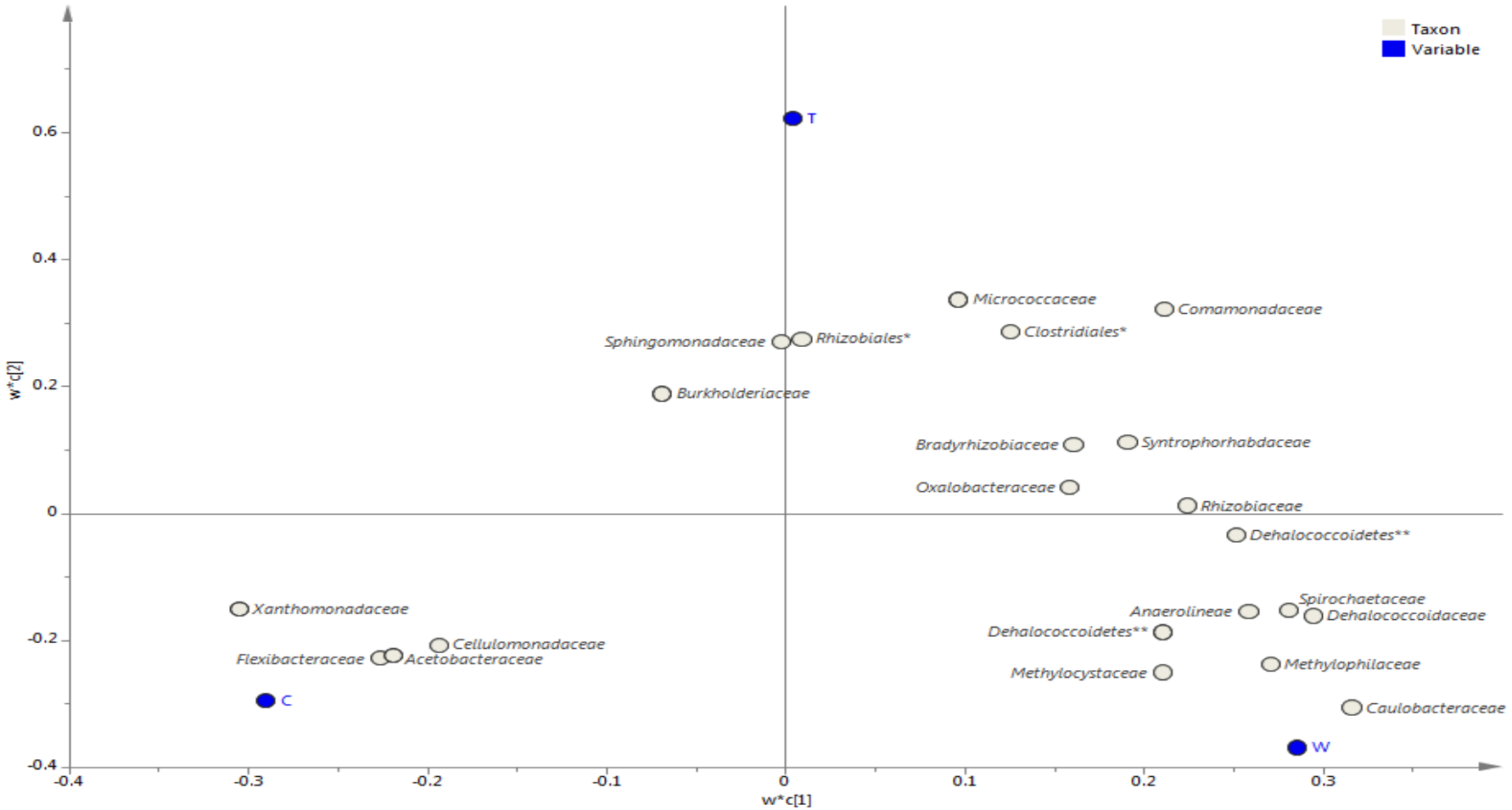


Figure 6.9: PLS-DA loading plot shows the relationship between taxa and environmental variables C, T and W. C = cold; T = transition; W = warm. Taxa that situated in the area close to the environmental variables suggest significant correlation while taxa located further from the variables indicate weaker relationship. Taxon is described at family level. * is assigned to taxon that has insufficient information to be called at family level. Taxon* = order level, Taxon**= class level, Taxon***= phylum level. Taxa with OTU counts <3 % are grouped as 'Others'.

6.4. Discussion:

Our DGGE analysis revealed that the bacterial communities in the warmer sediment deposits were more diverse than bacterial communities in the colder ecosystem sediments. On the other hand, RDA analysis based on bacterial DGGE profiles showed no significant relationship between climatic conditions and bacterial communities' structures. Instead, RDA analysis revealed that sediment depth (p = 0.002) and sulphur content (p = 0.038) were significantly related to bacterial communities' structures during a transition from a colder to a warmer climate. Similarly, bacterial community structure also demonstrated significant correlation to sulphur contents during both warmer and colder climates, when the transition period was not examined. Temperature has been suggested to be one of the primary factors that can impact on bacterial growth (Felip *et al*. 1996, Gurung and Urabe, 1999). However, the effect of temperature on sediment bacterial community is not necessarily a direct effect on the lake waters. Hall *et al*. (2008) found that the effect of temperature will influence the available of resource pool which will ultimately change the bacterial community composition and therefore, bacteria-mediated biogeochemical processes. Other works have reported that the mineralisation of organic carbon in lake sediments exhibits a strongly positive relationship with temperature, suggesting that warmer water temperatures lead to more mineralisation and less organic carbon burial (Gudasz *et al*., 2010).

As revealed by DGGE analysis, the significance of sulphur may suggest the importance of bacterial sulphur cycling in the old sediments of Lake Suigetsu. Studies have reported that in aquatic sediments, sulphur cycling is primarily driven by both reductive and oxidative bacterial processes (Jørgensen, 1982, 1990; Kondo *et al*., 2000). However, in the deep water layers and sediments, reduced diffusion of oxygen into the lake bottom ensures sulphate reduction predominante (Li *et al*., 1999a; Holmer and Storkholm, 2001). In the anoxic sediments, the concentration of sulphate is the key controlling factor of sulphate reduction rate (Sinke *et al*., 1992). Under anaerobic conditions, sulphate-reducing bacteria (SRB) reduce sulphate (SO_4^{2-}) to hydrogen sulphide (H_2S) and organic sulphur is formed (Holmer and Storkholm, 2001). A study by Li *et al*. (1999a) has detected high sulphate reduction rate from spring to summer and decreased rate from autumn to winter in Lake Kizaki, Japan. The variation in sulphate reduction rate with seasonal change was identified to have positive correlation

with the sulphate contents in the sediment (Li *et al*., 1999a). The sulphur contents in Lake Suigetsu's sediments on average have been shown to increase in order of sequence from cold environment (0.12), transition period (0.20) to warmer climate (0.32) (Table 6.5, Figure 6.10). This phenomenon is likely to suggest that the augmentation of sulphur during warmer climate may result from the increased sulphate reduction rates when climatic condition became warmer, therefore, the changed in bacterial communities' structures (i.e. SRB populations).

Sediment depth can be another factor that can alter the bacterial communities' structures. Each layer of laminated sediment represents different seasonal deposition and therefore is climate-related. In this case, sediment depth may relate to differences in climate and nutrient concentrations during the time of sediment deposition. Pearson correlation analysis demonstrated that bacterial diversity is significantly negatively correlated with sediment depth and iron (III) mineral while positively correlated with TOC, TN, sulphur and $BioSiO_2$ when climatic condition changed from colder ecosystem to warmer environmental condition.

Table 6.5: Characterisation of bacterial community profiles of Suigetsu core with increasing depth

Core SG06-A09	Age (BP)	Climate Condition	Composite depth (cm)	Total N	Total OC	Sulphur	$BioSiO_2$	Fe_2O_3
56	14400	Warm	1696.10	0.63	4.81	0.34	34.79	8.93
57	14411		1697.12	0.69	5.80	0.37	38.31	7.67
58	14421		1698.15	0.69	6.05	0.33	41.83	10.25
59	14434		1699.17	0.66	5.66	0.35	32.71	10.53
60	14445		1700.19	0.70	6.30	0.28	37.89	9.05
61	14458		1701.22	0.70	6.19	0.33	-	-
62	14469		1702.24	0.67	5.56	0.29	38.70	8.62
63	14481		1703.26	0.63	5.13	0.24	41.06	9.68
			Mean	0.67	5.69	0.32	37.90	9.25
64	14493	Transition	1704.28	0.70	5.99	0.34	35.56	10.90
65	14504		1705.31	0.72	6.42	0.30	33.71	10.78
66	14516		1706.33	0.69	6.11	0.35	31.90	11.18
67	14531		1707.35	0.65	5.41	-	33.37	11.88
68	14544		1708.38	0.73	6.23	0.34	30.29	10.76
69	14558		1709.40	0.64	5.81	0.24	32.63	11.44
70	14572		1710.41	0.68	6.03	0.26	35.84	10.57
71	14588		1711.43	0.71	5.58	0.24	34.54	10.30
72	14604		1712.44	0.64	5.33	0.23	31.05	11.19
73	14618		1713.46	0.64	5.59	0.24	35.60	10.07
74	14630		1714.47	0.64	5.49	0.22	25.99	11.64
75	14642		1715.49	0.61	4.93	0.20	35.16	8.56
76	14653		1716.50	0.53	4.28	0.19	32.65	10.53
77	14664		1717.47	0.62	5.26	0.20	28.50	13.37
78	14679		1718.43	0.54	5.99	0.21	26.66	10.50
79	14695		1719.40	0.48	4.77	0.15	23.22	11.95
80	14707		1720.50	0.50	4.39	0.14	17.92	8.86
81	14719		1721.40	0.55	4.35	0.15	-	-
82	14728		1722.30	0.42	3.63	0.13	28.39	12.73
83	14740		1723.20	0.60	5.78	0.21	22.27	12.63
84	14751		1724.10	0.59	5.59	0.20	19.50	12.05
85	14762		1725.00	0.61	4.94	-	24.75	14.84
86	14775		1725.90	0.71	5.86	0.20	19.75	11.98
87	14786		1726.80	0.58	4.64	0.15	19.45	12.99
88	14799		1727.70	0.52	3.87	0.13	16.76	11.01
89	14809		1728.60	0.50	3.76	0.13	14.56	10.15
90	14824		1729.63	0.48	3.60	0.12	18.88	11.31
91	14837		1730.67	0.49	3.76	0.15	12.79	12.63
92	14852		1731.70	0.53	4.75	0.16	18.54	14.62
93	14865		1732.70	0.54	5.16	0.16	19.10	12.83
94	14881		1733.70	0.54	4.60	0.15	18.16	14.41
95	14895		1734.70	0.55	4.63	0.15	16.34	15.47
			Mean	0.59	5.08	0.20	25.61	11.75
96	14910	Cold	1735.70	0.53	4.44	0.13	15.97	10.04
97	14925		1736.70	0.52	4.22	0.12	16.33	14.22
98	14939		1737.70	0.52	4.19	0.12	17.33	10.96
99	14952		1738.70	0.53	4.30	0.12	14.86	9.84
100	14967		1739.70	0.56	4.55	0.12	13.39	11.72
101	14983		1740.70	0.54	4.10	-	19.72	12.19
			Mean	0.53	4.30	0.12	16.27	11.50

Composite depth is taken from the midpoint of each of the 1 cm^3 sediment sample. Each sediment age represents the age at the midpoint of the composite depth. Total N = total nitrogen, Total C = total organic carbon, $BioSiO_2$ = biogenic silica, Fe_2O_3 = Iron (III) oxide/ferric oxide. '-' indicates data unavailable.

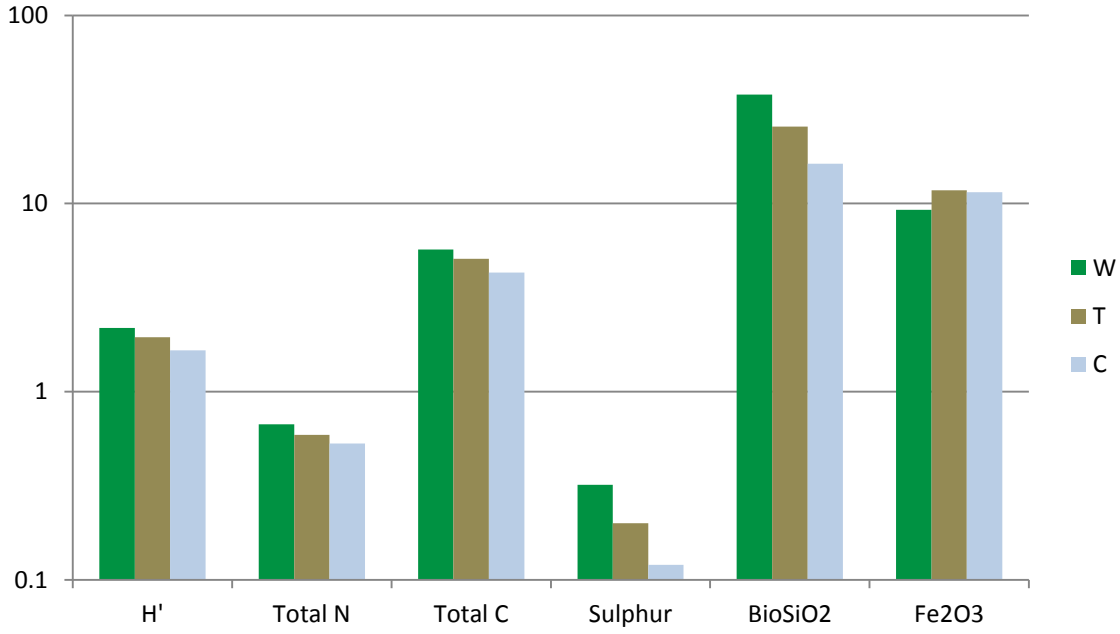


Figure 6.10: Bar chart of bacterial diversity and environmental variables across climatic changes. W = warm, T = transition, C = cold. H' = bacterial Shannon Weiner Index. Total N = total nitrogen, Total C = total organic carbon, $BioSiO_2$ = biogenic silica, Fe_2O_3 = Iron (III) oxide/ferric oxide.

The rates of organic matters accumulated and the rate of anaerobic microbial processes of sulphate-reduction (Chrzanowskil and Grover, 2001; Li *et al*., 1999a) and denitrification (Meckler *et al*., 2007) in the sediments have been reported to have positive correlation with temperature (Bergström *et al*., 2010; Hodell and Schelske, 1998). The rise in temperature will increase the microbial metabolic rate, mineralisation rate and subsequently the microbial redox reactions in sediments (Lehtoranta, 2003). Therefore, in this case, the significance of TOC, TN, sulphur and BioSiO2 may indicate that the shift in climatic condition from colder to warmer episode has resulted in the increased input of organic matter, hence affected the bacterial diversity.

In contrast, Pearson correlation showed that iron (III) oxide has a negative correlation to bacterial diversity. In the anoxic lake sediments, the accumulated iron (III) oxides can be reduced either biological or non-biologically to iron (II) (Lehtoranta, 2003; Lovley *et al.* 1990) and the reduction of iron (III) is said to have significant contributions to the oxidation of organic matters in aquatic environments (Lehtoranta, 2003; Lovley, 1991). Under anaerobic condition, the poorly crystalline Fe (III) oxides are readily reducible to iron (II) by Fe-reducing bacteria provided that there is direct contact between bacteria and iron (III) compounds (Lovley, 1991; Lovley *et al.* 1990). Likewise, as discussed before, microbial metabolic rate is temperature-dependent and

so, when climatic condition becomes warmer, bacterial metabolic rate will increase and more iron (III) oxide compounds will be reduced, lowering iron (III) oxide in the sediments.

Heino *et al*. (2009) indicated that the decreased and increased levels of nutrients entering the freshwater ecosystems are climate-dependent. In particular, in naturally oligotrophic lakes, the increasing of nutrient levels may represent the beginning of the eutrophication process and hence will lead to the increased levels of biodiversity. On the contrary, in eutrophic (nutrient-rich) freshwater ecosystems that result from either natural or anthropogenic causes, the increased nutrient levels may often lead to reductions in biodiversity (Heino *et al*., 2009).

In order to further distinguish the taxa in colder sediment deposits from warmer sediments, several samples covering these ecosystems including the transition period were selected for metagenomics sequencing. The re-analysis of CCA analysis of DGGE data and RDA analysis of metagenomics data revealed that $BioSiO_2$ has a significant relationship with the bacterial communities' structures. Recent studies indicated that silicon is the second most abundant element in lake sediments (Shan *et al*., 2011) and this element is usually preserved in sediments in the form of biogenic silica (BSi), produced by diatoms and chrysophytes (Shan *et al*., 2011). The supply of silica is important for diatom growth which is largely maintained by the recycling (dissolution) of biogenic silica (Conway *et al*. 1977).

Measurements of BSi have been widely used in acquiring past climatic information on glacial-interglacial cycles (Swan and Mackay, 2006). This is thought to be plausible as the change in lake productivity is interrelated with nutrients availabilities and water surface temperature (Hu *et al*., 2003). According to several studies, high BSi values are shown to correspond with warm and humid climatic conditions while low values indicate cold and dry climates (Swann *et al*., 2006). Therefore, the strong correlation between BSi and bacterial communities' structures revealed by the metagenomics analysis suggests that the changes in climatic conditions may have affected the bacterial composition. Nevertheless, the physical interactions between BSi and bacterial community have not been reported.

On the other hand, data (Figure 6.7 and 6.9) based on the OTU counts of taxa at order and family level have shown that the taxa associated with the colder environment were very different from those in the transition and warmer climatic conditions. Linström *et al*. (2005) also found similar findings when comparing the distribution of 15 typical freshwater bacterial groups from 15 diverse lakes in northern Europe. They noticed that the *Alphaproteobacteria* are associated with warmer temperature while *Betaproteobacteria* are related to colder climatic conditions. PLS-DA analysis also demonstrated that *Caulobacteraceae* from phylum *Alphaproteobacteria* predominate in warmer sediment deposits but *Xanthomonadaceae*, which belong to phylum *Gammaproteobacteria*, are prevailing in colder sediment deposits. In fact, studies have reported the presence of *Alphaproteobacteria* in several hot ecosystems such as the geothermal ecosystem of the Blue Lagoon in Iceland (Petursdottir *et al*., 2009) and the acidic hot spring in the Colombian Andes (Jiménez *et al*., 2012). The *Xanthomonadaceae* of phylum *Gammaproteobacteria* have also been reported to be found in the Arctic ice shelf in Canada as one of the taxa that can adapt to an extreme cold environment (Bottos *et al*., 2008).

In addition, the difference in bacterial taxa when climatic condition changes could be either due to the adaptation of bacterial community to new climatic conditions or was simply an effect of depth (Fredrickson *et al*., 1997; Rebata-Landa and Santamarina, 2006). Bacterial diversity in this case would change as bacterial taxa that cannot adapt quickly to the abrupt changes in climatic condition will be replaced with those best adapted ones (Hartley *et al*., 2008; Rinnan *et al*., 2009). According to Bárcenas-Moreno *et al*. (2009), this is a selective pressure which can result in cell death and meanwhile creating a competitive environment for bacteria that can tolerate higher temperature. Hence, bacteria with higher tolerance can rapidly out-compete the original community. This can also imply that *Rhizobiales* and *Burkholderiales* (at order level) that have been detected throughout the sediment samples for metagenomics analysis may be the taxa that can best adapt to the ecological transition from colder to warmer climatic condition.

Bacterial communities inhabiting stable environmental conditions are more sensitive to changes of temperature in comparison to those in a more variable environment (Waldrop and Firestone, 2006; Rinnan *et al*., 2009). Former studies of Lake Suigetsu have demonstrated that the water temperature below 10 m was stable throughout the year regardless of seasonal change (Kondo and Butani, 2007; Kondo *et*

al., 2000; Mori *et al*., 2013). So, the gradual increased in the annual constant temperature of the stable anoxic water and lake sediment, may be more responsive to the changes of atmospheric temperature from glacial condition to warmer climate and therefore may affect the sediment bacterial community. Heino *et al*. (2009) also indicated that the degree to which increased nutrient inputs and biodiversity in association with climate change is dependent on the starting condition of an ecosystem. The increase in nutrient inputs may increase the diversity of different organism groups in oligotrophic lakes while in eutrophic lakes the levels of biodiversity may be reduced due to excess nutrients.

6.5. Conclusions:

In summary, the shift in climatic conditions from glacial to a warmer atmospheric condition may have resulted in the measurable variation of organic matter deposition in the sediment alongside the changed in bacterial communities' structures and diversity. This study indicates that nutrient concentrations vary with inferred environmental temperature and climate (derived from pollen data) and bacterial communities which can tolerate and adapt rapidly to these changes may outcompete the original community. The OTU counts at family level as revealed by metagenomics analysis have identified possible taxa that can correlate to climate. *Caulobacteraceae* can probably represent the taxa for warmer climate while *Xanthomonadaceae* may be representative taxa of colder climate. Further work could also focus at sediments around 14,500 yr BP as according to Yasuda *et al*. (2004), ecological transitions may take approximately 500 to 800 years to completely establish.

Chapter 7 Culture-dependent Analysis of Microbial Diversity

7.1. Introduction

Microorganisms are the most highly diverse and most abundant organisms, making up approximately 60% of the earth's biomass (Fierer and Jackson 2006; Shade *et al*., 2012; Singh *et al*. 2009; Whitman *et al*., 1998). Terrestrial habitats such as soils contain an enormous amount of microbial cells (*ca*. 4–5 × 10^{30}) which is vastly more abundant than those originating from the oceans (~1.2 × 10^{29} microbial cells) (Singh *et al*., 2009). Studies showed that the microbial community structure and function in these ecosystems are vital (Fierer and Jackson 2006) as microorganisms in these habitats are known to play important roles in various biogeochemical processes mainly in the mineralisation of organic matter (Jørgensen 1982) and the cycling of essential elements (i.e. carbon, nitrogen, phosphorus, and sulphur) (Bhattarai *et al*., 2012; Holmer and Storkholm 2001; Ohkouchi *et al*., 2005; Pinhassi *et al*., 2006).

Currently, there are 61 distinct bacterial phyla (Hugenholtz *et al*., 2009) and 54 species of *Archaea* that have been cultured to date and this represents only a small fraction of the total diversity (Auguet *et al*., 2010). The use of molecular techniques has become increasingly popular to study the community structure and diversity of microbial populations compared to the conventional culture-dependent approaches (Nannipieri *et al*., 2003). However, despite the fact that conventional culturing techniques are time-consuming, laborious and can recover only a small portion of microbial diversity from an environmental sample (Daniel, 2005; Nannipieri *et al*., 2003), studies have suggested that the combined used of culture-based and molecular methods are useful in providing a more comprehensive overview of the microbial community (Edenborn and Sexstone, 2007). Each technique has been demonstrated to be capable of recovering distinct bacterial taxa. The high GC Gram-positive bacteria for instance, are mainly better recovered through culture-dependent techniques (Smit *et al*., 2001) while molecular approaches deliver a better profile of *Acidobacteria* (Barns *et al*., 1999). In addition, culture-based techniques are also said to be essential in estimating microbial ecological roles in ecosystems (Tamaki *et al*., 2005) and only through the

isolation of bacteria, can an understanding of microbial physiology from a complex environment be achieved (Nannipieri *et al*., 2003; Zengler *et al*., 2002).

Former microbial studies of Lake Suigetsu that have been carried out were solely based on molecular techniques such as competitive PCR, cloning and sequencing (Kondo and Butani, 2007; Kondo *et al*., 2006), real-time PCR (Kondo *et al*., 2008) and PCR-DGGE of bacterial 16S rRNA genes (Kondo *et al*., 2009). Therefore, apart from the molecular techniques that have been presented in the previous chapters, culture-dependent techniques will be employed in this study to enhance the overview of microbial diversity in the sediments of Lake Suigetsu.

The changes of microbial ecology, activity, community structure and its diversity in soil are mainly affected by various essential environmental factors, including carbon and energy sources, mineral nutrients, growth factors, ionic composition, water availability, temperature, pressure, oxygen levels and pH (Nannipieri *et al*., 2003, Nielsen *et al*., 2003). In the present study, the area of focus was directed at the microbial diversity before and after salinity shifts using an improved cultivation approach of dispersion and differential centrifugation (DDC) (Hopkins *et al*., 1991), alongside sequencing techniques, aiming to recover and determine 'culturable' bacterial species in the sedimentary records of Lake Suigetsu from both the brackish (335 BP) and freshwater sediments (6,860 and 10,911 BP). This area was targeted as studies have indicated that salinity can control microbial compositions (Oren, 2002; Jiang *et al*., 2006) through species replacement process (Nielsen *et al*., 2003; Wu *et al*. 2006), hence can yield a significant difference in microbial compositions from different lake conditions. In addition, representative isolates which have been successfully recovered will also be characterised on both macro and micro morphology, following by the comparison of microbial data to those detected from the molecular techniques.

7.2. Experimental strategy:

Sediment samples representing the brackish and freshwater regions were targeted. The DDC technique was utilised to disperse and segregate bacteria from sediment soil aggregates. Serial dilution (to 10^{-4}) was performed and nutritious TPA and MA media were used to recover sediment bacteria at 25°C incubation temperature. Basic macro- and micro-morphology characterisations were performed for all representative isolates.

In order to further identify the possible species identity, the genomic DNA of each representative isolates was extracted and the 16S rRNA genes were amplified. The phylogenetic relationships between isolates and their closest type strains was determined using MEGA 5.0 software (Tamura *et al*., 2011).

7.3. Results:

7.3.1. Isolation and Enumeration of Taxa

Through the use of DDC, less selective media MA and TPA, as well as a lower temperature at 25°C for incubation, a total of 2.53×10^7 cfu/g with 158 morphologically different isolates (Table 7.1) were successfully recovered from brackish sediment sample (A01) (Figure 7.1a; 7.1b; Table 1), while 7.78×10^8 cfu/g of colonies were isolated from the freshwater sediment samples of B (N) 05 (with 116 morphological different isolates; Table 7.1) (Figure 7.1c; 7.1d) and B (N) 07 (with 49 morphological different isolates; Table 7.1) (Figure 7.1e; 7.1f). The different fractions obtained during the multiple stages of the DDC protocol, supernatants A, B, C and D, respectively have also resulted in the diversification of bacterial taxa. Table 7.1 illustrates a summary of various representative isolates that were recovered from using both the MA and TPA media from different fractions. Fraction A was the first supernatant collected after the treatment using mild detergent (sodium cholate) whereas fraction B was obtained by the treatment of attenuated physical disruption with mild ultrasonication. Fraction C was attained by the treatment of ionic shock with cold distilled water while fraction D was the last faction of supernatant containing ¼ strength Ringer's solution. Table 7.1 demonstrates that there were higher numbers of isolates that were recovered on TPA medium compared to the MA medium. The use of combined physiochemical treatments in the successive stages of the DDC protocol has demonstrated the ability to recover different bacterial taxa from different stages. Although the numbers of representative isolates calculated for both the freshwater and brackish sediments are about the same, the bacterial diversity observed for the freshwater sediments is greater than the brackish sediment, with highly diverse *Actinobacteria* taxa (Table 7.1; 7.2).

Table 7.1 Recovery of bacteria at various stages of DDC protocol

MA			
Freshwater sediment			**Brackish sediment**
Fraction	**B(N) 05 (6860 BP)**	**B(N) 07 (10911 BP)**	**A01(335 BP)**
A	*Micrococcus* F179M	*Arthrobacter* F65M, F88M	*Bacillus* S3M, S4M, S10M, S14M, S24M, S32M, S33M, S36M, S37M, S39M, S47M, S52M, S54M, S65M, S67M, S66M, S68M, S70M, S72M, S77M, S78M, S81M, S91M, S96M, S362M
		Corynebacterium F25M, F26M	*Paenibacillus* S22M, S41M, S44M, S48M, S49M, S50M, S56M, S80M, S84M, S88M, S89M, S95M
		Jeotgalicoccus F24M	*Paucisalibacillus* S46M
		Kocuria F28M, F27M	*Oceanobacillus* S79M
			Virgibacillus S58M
B	*Bacillus* F45M, F183M	*Bacillus* F91M, F61M	*Bacillus* S98M, S99M, S102M, S111M, S128M, S134M
	Kocuria F216M	*Arthrobacter* F30M, F32M	*Paenibacillus* S101M, S103M, S104M, S115M, S116M, S120M
	Micrococcus F215M	*Micrococcus* F29M, F62M, F90M	*Virgibacillus* S136M
		Rhodococcus F31M, F42M, F152M	
C	*Arthrobacter* F192M	*Caryophanon* F53M	*Bacillus* S138M
	Dermacoccus F184M, F186M, F187M, F188M, F189M, F190M, F191M	*Paracoccus* F52M	*Paenibacillus* S139M, S140M
	Kocuria F182M	*Arthrobacter* F51M	*Janibacter* S142M
	Micrococcus F214M	*Frigobacterium* F185M	
		Knoellia F93M	
		Micrococcus F33M	
		Ornithinimicrobrium F50M	
		Staphylococcus F63M, F64M	
D	*Bacillus* F217M	*Pseudomonas* F55M	
	Kocuria F180M, F204M	*Micrococcus* F54M, F57M	
TPA			
A	*Pseudomonas* F128T	*Enhydrobacter* F60T, F86T	*Bacillus* S145T, S146T, S150T, S168T, S170T, S172T, S176T, S179T, S182T, S199T, S200T, S207T, S217T, S221T, S244T, S250T, S251T, S254T, S255T, S256T, S257T, S268T, S297T, S320T
	Arthrobacter F14T, F101T	*Arthrobacter* F70T, F84T	*Paenibacillus* S144T, S148T, S149T, S152T, S154T, S157T, S159T, S164T, S178T, S187T, S190T, S191T, S192T, S197T, S202T, S204T, S211T, S219T, S220T, S226T, S227T, S228T, S229T, S231T, S232T, S238T, S239T, S240T, S242T, S248T, S249T, S252T, S259T, S260T, S261T, S265T, S266T, S267T, S269T, S270T
	Dermacoccus F124T, F149T	*Dermacoccus* F72T	
	Kocuria F125T	*Leifsonia* F71T, F83T	
	Micrococcus F111T	*Staphylococcus* F85T	
	Staphylococcus F103T, F107, F119T, F123T, F133T		
	Streptomyces F127T		

Fraction	B(N) 05 (6860 BP)	B(N) 07 (10911 BP)	A01(335 BP)
B	*Bacillus* F105T, F135T, F136T	*Arthrobacter* F75T, F76T, F79T, F80T, F87T	*Bacillus* S273T, S282T, S290T, S302T, S308T, S315T, S321T, S334T, S354T
	Lysinibacillus F13T	*Citricoccus* F77T, F95T	*Brevibacillus* S301T
	Brevundimonas F139T	*Leifsonia* F96T	*Paenibacillus* S271T, S276T, S278T, S279T, S281T, S283T, S285T, S287T, S291T, S294T, S295T, S300T, S306T, S318T, S329T, S332T, S357T
	Arthrobacter F109T, F113T	*Micrococcus* F82T, F73T	
	Dermacoccus F142T	*Mycobacterium* F59T	
	Kocuria F112T, F126T, F146T	*Rhodococcus* F58T, F74T	
	Kytococcus F134T		
	Micrococcus F129T, F131T		
	Staphylococcus F106T, F108T, F140T, F147T, F197T		
	Streptomyces F114		
C	*Bacillus* F137T	*Staphylococcus* F68T	*Bacillus* S339T, S347T, S349T
	Brevundimonas F117T		*Paenibacillus* S336T, S338T, S342T, S343T, S345T, S346T, S351T
	Dermacoccus F145T, F154T, F203T, F218T, F219T, F220T		*Sporosarcina* S353T
	Janibacter F158T		
	Kocuria F157T, F193T, F198T, F200T, F208T		
	Micrococcus F110T, F120T, F144T, F155T, F207T		
	Staphylococcus F118T, F143T, F148T, F205T, F206T		
D	*Bacillus* F194T, F213T	*Pseudomonas* F69T	
	Brevundimonas F94T, F115T, F141T, F150T, F202T		
	Pseudomonans F169T, F172T		
	*Brevibacterium*F201T		
	Dermacoccus F116T, F122T, F156T, F195T, F170T, F212T		
	Dietzia F130T		
	Kocuria F132T, F153T, F161T, F165T, F167T, F175		
	Microbacterium F196T		
	Micrococcus F159T, F173T, F176T, F177T, F199T, F210T, F211T		
	Staphylococcus F162T, F164T, F209T		
Residue	*Pseudomonas* F171T		
	Micrococcus F160T		
	Kocuria F168T		

Table shows the difference in representative isolates that were recovered at different stages of DDC protocols. Fraction A: first supernatant collected after the treatment using mild detergent (sodium cholate); B = obtained after the treatment with mild ultrasonication; C = attained after the treatment of ionic shock with cold distilled water; D = the last faction of supernatant with ¼ strength Ringer's solution. Residue = the remaining mixture.

Table 7.2: Total number of representative isolates detected from brackish A01 and freshwater B (N) 05 and B (N) 07 sediments and their closely related species.

Taxa recovered	Brackish sediment	Freshwater sediments	
Gram positive	**A01** (335 BP)	**B (N) 05** (6860 BP)	**B (N) 07** (10911 BP)
Bacillus	68	6	3
Brevibacillus	1	-	-
Caryophanon	-	-	1
Jeotgalicoccus	-	1	-
Lysinibacillus	-	1	-
Oceanobacillus	1	-	-
Paenibacillus	84	-	-
Paucisalibacillus	1	-	-
Sporosarcina	1	2	-
Staphylococcus	-	18	4
Virgibacillus	2	-	-
Gram negative			
Brevundimonas	-	7	-
Enhydrobacter	-	2	-
Paracoccus	-	1	-
Pseudomonas	-	4	2
Actinobacteria			
Arthrobacter	-	5	12
Brevibacterium	-	1	-
Citrococcus	-	-	2
Corynebacterium	-	-	2
Dermacoccus	-	22	1
Dietzia	-	1	-
Frigobacterium	-	-	1
Janibacter	1	1	-
Knoellia	-	-	1
Kocuria	-	20	2
Kytococcus	-	1	-
Leifsonia	-	-	3
Microbacterium	-	1	-
Micrococcus	-	20	8
Mycobacterium	-	-	1
Ornithinimicrobium	-	-	1
Rhodococcus	-	-	5
Streptomycetes	-	2	-
Total:	**158**	**116**	**49**

Table shows the number of representative isolates recovered from different sediment conditions which can be categorised into three major groups, the Gram positive, Gram negative and the *Actinobacteria*.

Subsequently, colonial pigmentation was determined based on National Bureau of Standards (NBS) Colour Name Charts (Kelly 1958; NBS 1964), along with colonial form, elevation and margins (Appendix 12a, 12b, 12c). The comparison of macro morphology of the isolates recovered from brackish sediment demonstrated that they possessed very different characteristics to the strains isolated from the freshwater sediments. A basic Gram-staining analysis was also carried out to ascertain if the isolates were Gram-positive or Gram-negative isolates. The results revealed that majority of the strains recovered had retained the crystal violet - iodine complex (mainly coccus/short rod), indicating Gram-positive bacteria were predominant in both the fresh and brackish sediments than the Gram-negative bacteria. The analysis of micro-morphology also revealed that some of the Gram-positive isolates retained endospores. Among the Gram-positive isolates recovered, Gram-positive *Actinobacteria* were easily recognisable with their distinctive macro-morphology of filamentous colonies, slightly embedding into the agar surface and possessing a unique smell of soil (Figure 7.1e). Other distinct representative isolates that were able to produce diffusible pigments were also detected which are shown in Figure 7.1g. Other tests such as acid-fast stain was also performed selectively for *Actinobacteria* isolates but none of the selected isolates retained carbol fuchsin dye in the bacterial cell wall which indicated that the strains were non-acid fast bacteria.

The lake bottom of Lake Suigetsu is anoxic, therefore, anaerobic cultivation using Nitrate Minimal Media (NMS) and NMS-Artificial Sea Salt (NMS-ASS) (Dworkin *et al*., 2006) was attempted initially using candle jars for incubation for sediment sample B (N) 05. All DDC fractions were plated on both media in duplicate and serially diluted to 10^{-2}. The incubation period for the growth of anaerobes on both media was more extended than the aerobic culture as the anaerobes took at least a minimum period of 8 weeks to grow. On the 8^{th} week of incubation, isolates were observed to grow on only the NMS-ASS medium (Figure 7.1h) but not the NMS medium. However, these anaerobic isolates were unable to be subcultured on fresh plates due to the fact that they took more than 8 weeks to grow on the fresh medium and with very little biomass produced. An alternative of using anaerobic Bug Box for cultivation and incubation was also attempted. Unfortunately, there were technical issues resulting in insufficient data.

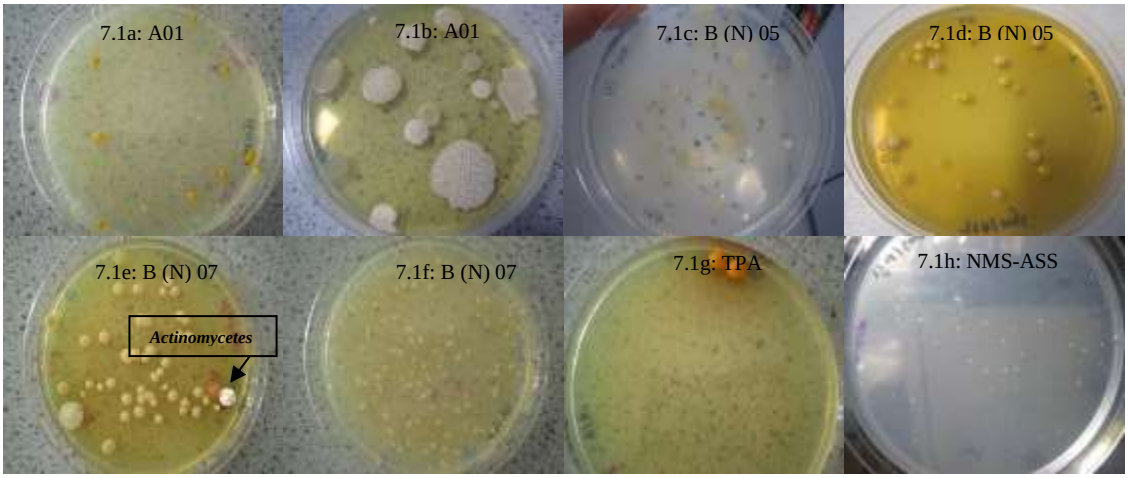


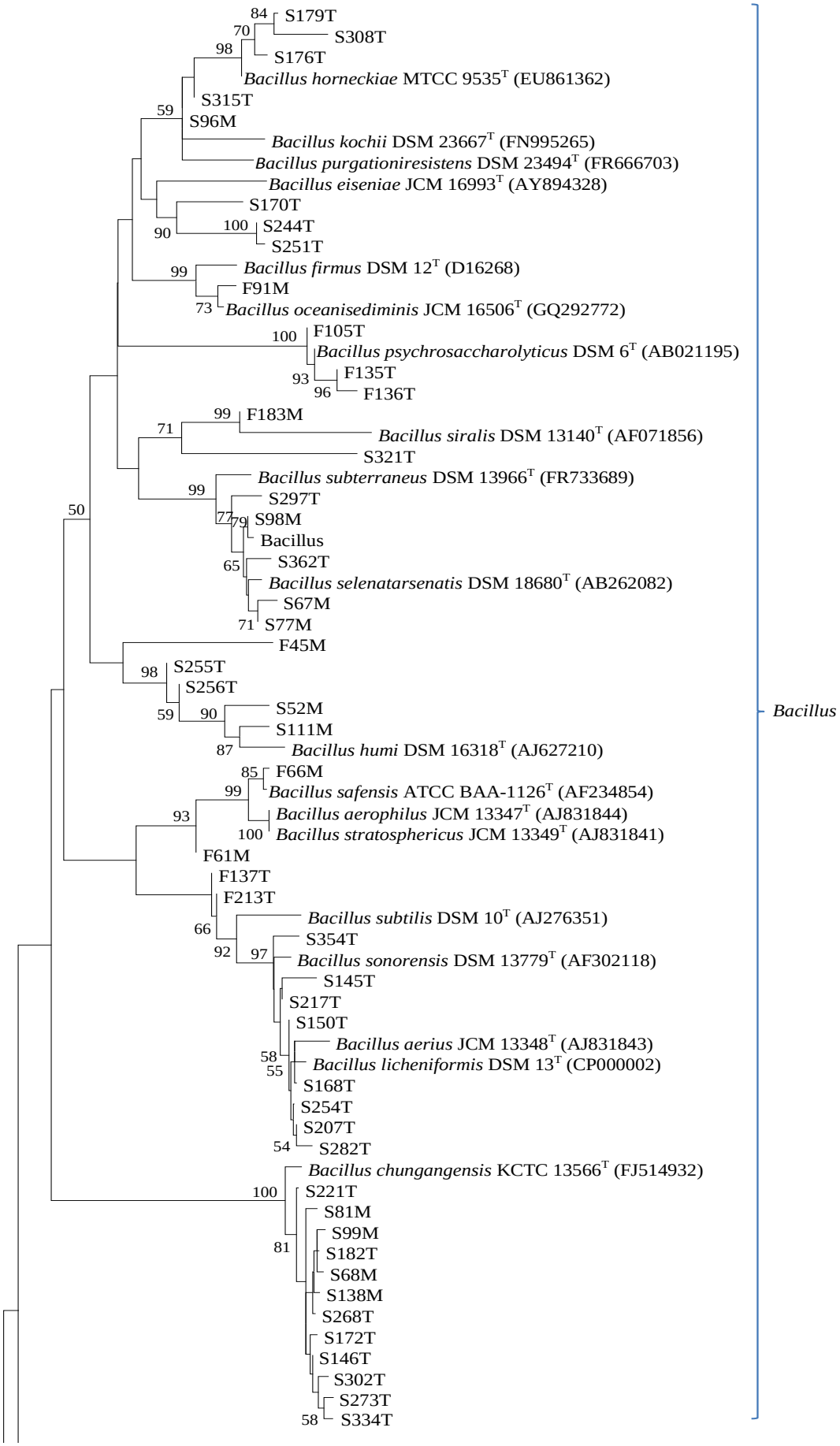
Figure 7.1: Culture plates incubated at 25°C for minimum 7 days with nystatin supplemented in these media except for 7.1h which has been incubated anaerobically at 25°C for at least 8 weeks. Figure 7.1a and 7.1b = isolates from brackish sediment A01, formed on TPA and MA media (both isolates recovered from supernatant A, dilution 10^{-1}), respectively; 7.1c to 7.1f = isolates recovered from freshwater sediments of B (N) 05 and 07. 7.1c = isolates on TPA medium from supernatant B, at dilution 10^{-1}; 7.1d = isolates on MA medium from supernatant C, at dilution 10^{-3}; 7.1e and 7.1f = both isolates from TPA and MA media were recovered from supernatant A, dilution 10^{-1}; 7.1g = isolates that produced diffusible pigments on TPA medium; 7.1h = isolates grown on NMS-ASS medium from core B (N) 05.

7.3.2. 16S rRNA gene sequencing and Phylogenetic analysis

Besides macro and micro morphology, 16S rRNA gene sequencing was also performed to further characterise and determine the relationship between morphologically different isolates which were recovered from TPA and MA media compared to reference bacteria. The forward and reverse sequences of the 16S rRNA gene, amplified by com 1 (CAG CAG CCG CGG TAA TAC) and com 2 (CCG TCA ATT CCT TTG AGT TT) primers, and were re-assembled using ABI MicroSEQ® ID Analysis Software v2.2. Ideally, the amplification using primers com 1 and 2 should yield *ca*. 1,400 base pairs (bp), however for some sequences the re-assembled 16S rRNA gene was less than 1400 bp due to the background contamination and the poor quality of the resulting sequences. The re-assembled 16S rRNA gene sequences were subsequently subjected to BLAST analysis (Altschul *et al*., 1990) for species identification. BLAST analysis revealed a total of 28 distinct bacterial taxa were recovered from the freshwater sediment samples while seven were identified from the brackish sediment sample (Table 7.2). There were 18 *Actinobacteria* taxa, six Gram-positive and four Gram-negative taxa recovered from the freshwater sediments, while there were only one actinobacterium alongside seven Gram-positive bacterial taxa in brackish sediment. Phylogenetic analysis was carried out based on the neighbour-joining algorithm (Saitou and Nei, 1987) and Jukes and Cantor model (1969) using MEGA software version 5.0 (Tamura *et al*., 2011) to identify the relationship and

similarities between each related isolates (Figure 7.2, 7.3 and 7.4). The topologies of the resultant trees were also evaluated using bootstrap analysis (Felsenstein, 1985) which calculated at 1000 replications based on neighbor-joining dataset.

For the Gram positive neighbour-joining tree (Figure 7.2), the representative isolates were clustered into various genera but mostly fell within the clades containing the genera *Bacillus* and *Paenibacillus*. Interestingly, all the representative isolates that fell within the *Paenibacillus* clade were isolated from the brackish sediment whereas the representative isolates that were analysed in the Gram negative neighbour-joining tree (Figure 7.3) comprised of isolates solely originated from freshwater sediments which were classified into three different genera; the *Paracoccus, Enhydrobacter* and *Pseudomonas*. Whilst, for the *Actinobacteria* neighbour-joining tree (Figure 7.4), most of the representative isolates were recovered from the freshwater sediments and among the *Actinobacteria* genera, *Arthrobacter, Dermacoccus, Kocuria* and *Micrococcus* have demonstrated to be the predominating taxa within the freshwater sediments. Table 7.2 summarises that the microbial diversity was generally higher in freshwater sediments than brackish sediment. In freshwater sediments, there were highly diverse *Actinobacteria* with only one member of *Actinobacteria* phylum recovered from the brackish sediment. Similarly, there was no Gram negative bacteria found in brackish sediment but plentiful Gram positive taxa such as the *Bacillus* and *Paenibacillus*.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S308T
S176T
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S315T
S96M
Bacillus kochii DSM 23667T (FN995265)
Bacillus purgationiresistens DSM 23494T (FR666703)
Bacillus eiseniae JCM 16993T (AY894328)
S170T
S244T
S251T
Bacillus firmus DSM 12T (D16268)
F91M
Bacillus oceanisediminis JCM 16506T (GQ292772)
F105T
Bacillus psychrosaccharolyticus DSM 6T (AB021195)
F135T
F136T
F183M
Bacillus siralis DSM 13140T (AF071856)
S321T
Bacillus subterraneus DSM 13966T (FR733689)
S297T
S98M
Bacillus
S362T
Bacillus selenatarsenatis DSM 18680T (AB262082)
S67M
S77M
F45M
S255T
S256T
S52M
S111M
Bacillus humi DSM 16318T (AJ627210)
F66M
Bacillus safensis ATCC BAA-1126T (AF234854)
Bacillus aerophilus JCM 13347T (AJ831844)
Bacillus stratosphericus JCM 13349T (AJ831841)
F61M
F137T
F213T
Bacillus subtilis DSM 10T (AJ276351)
S354T
Bacillus sonorensis DSM 13779T (AF302118)
S145T
S217T
S150T
Bacillus aerius JCM 13348T (AJ831843)
Bacillus licheniformis DSM 13T (CP000002)
S168T
S254T
S207T
S282T
Bacillus chungangensis KCTC 13566T (FJ514932)
S221T
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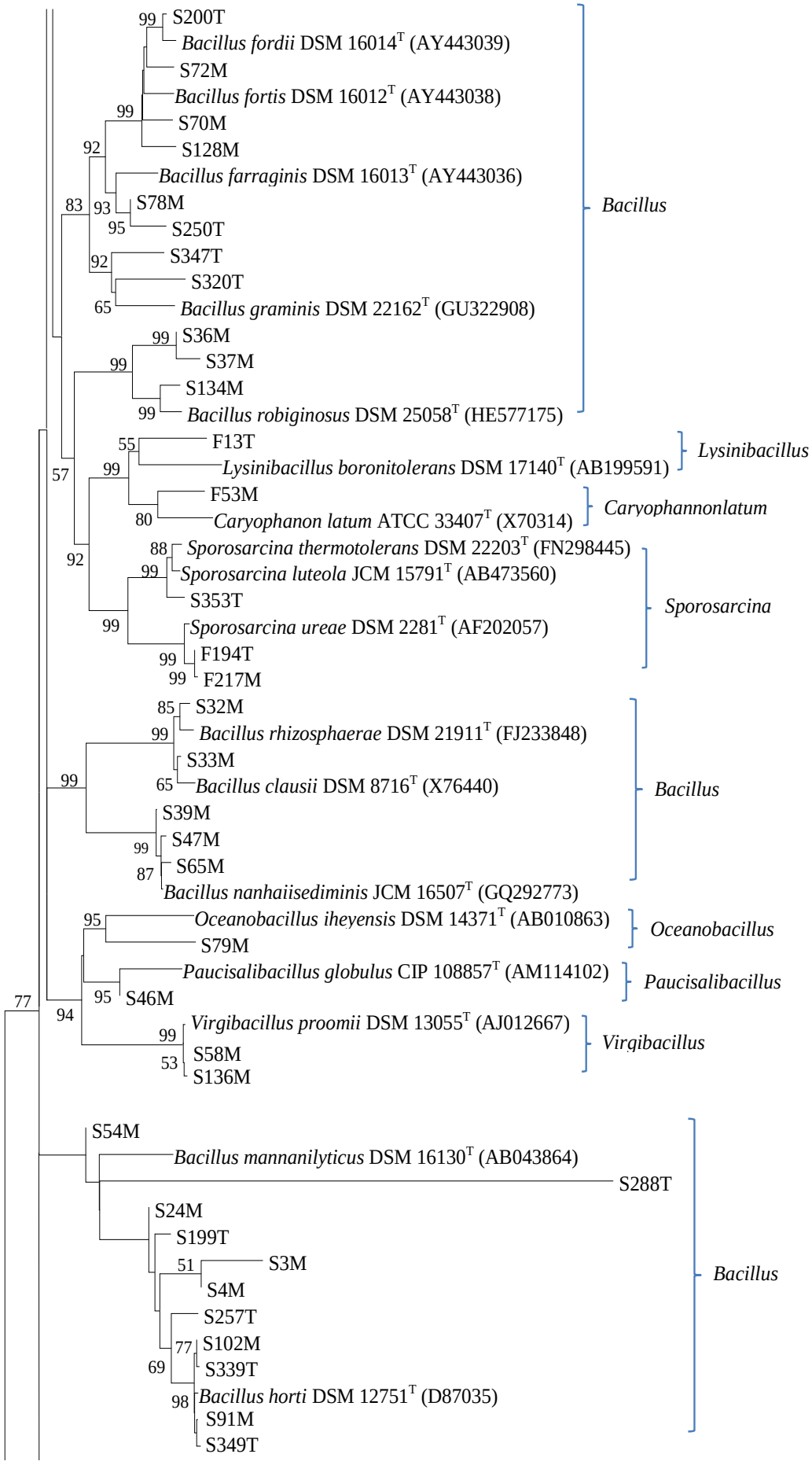
S302T
S273T
S334T
Bacillus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S72M
Bacillus fortis DSM 16012T (AY443038)
S70M
S128M
Bacillus farraginis DSM 16013T (AY443036)
S78M
S250T
S347T
S320T
Bacillus graminis DSM 22162T (GU322908)
S36M
S37M
S134M
Bacillus robiginosus DSM 25058T (HE577175)
Bacillus
F13T
Lysinibacillus boronitolerans DSM 17140T (AB199591)
Lysinibacillus
F53M
Caryophanon latum ATCC 33407T (X70314)
Caryophannonlatum
Sporosarcina thermotolerans DSM 22203T (FN298445)
Sporosarcina luteola JCM 15791T (AB473560)
S353T
Sporosarcina ureae DSM 2281T (AF202057)
F194T
F217M
Sporosarcina
S32M
Bacillus rhizosphaerae DSM 21911T (FJ233848)
S33M
Bacillus clausii DSM 8716T (X76440)
S39M
S47M
S65M
Bacillus nanhaiisediminis JCM 16507T (GQ292773)
Bacillus
Oceanobacillus iheyensis DSM 14371T (AB010863)
S79M
Oceanobacillus
Paucisalibacillus globulus CIP 108857T (AM114102)
S46M
Paucisalibacillus
Virgibacillus proomii DSM 13055T (AJ012667)
S58M
S136M
Virgibacillus
S54M
Bacillus mannanilyticus DSM 16130T (AB043864)
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S24M
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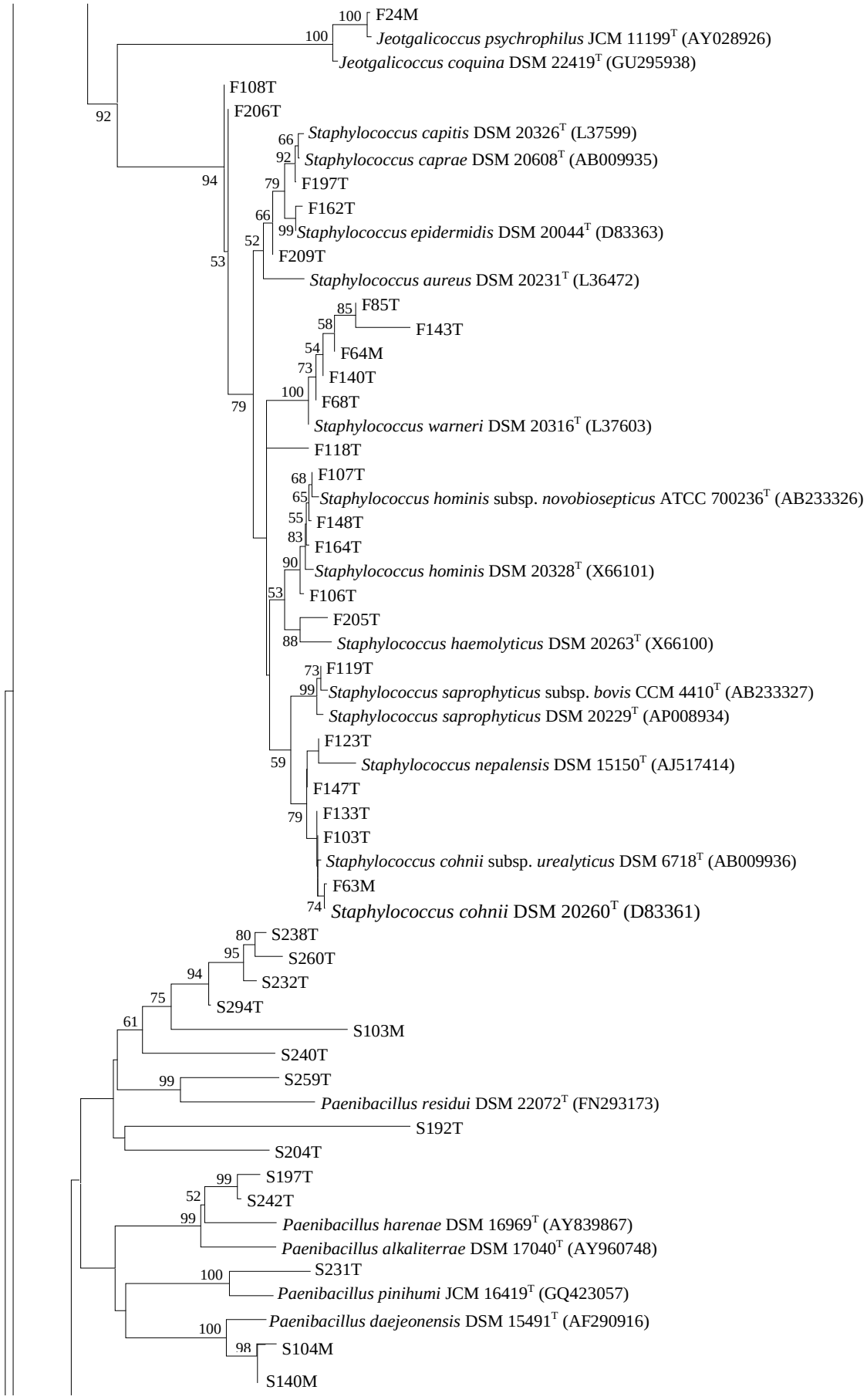
S257T
S102M
S339T
Bacillus horti DSM 12751T (D87035)
S91M
S349T
Bacillus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F108T
F206T
Staphylococcus capitis DSM 20326T (L37599)
Staphylococcus caprae DSM 20608T (AB009935)
F197T
F162T
Staphylococcus epidermidis DSM 20044T (D83363)
F209T
Staphylococcus aureus DSM 20231T (L36472)
F85T
F143T
F64M
F140T
F68T
Staphylococcus warneri DSM 20316T (L37603)
F118T
F107T
Staphylococcus hominis subsp. novobiosepticus ATCC 700236T (AB233326)
F148T
F164T
Staphylococcus hominis DSM 20328T (X66101)
F106T
F205T
Staphylococcus haemolyticus DSM 20263T (X66100)
F119T
Staphylococcus saprophyticus subsp. bovis CCM 4410T (AB233327)
Staphylococcus saprophyticus DSM 20229T (AP008934)
F123T
Staphylococcus nepalensis DSM 15150T (AJ517414)
F147T
F133T
F103T
Staphylococcus cohnii subsp. urealyticus DSM 6718T (AB009936)
F63M
Staphylococcus cohnii DSM 20260T (D83361)
S238T
S260T
S232T
S294T
S103M
S240T
S259T
Paenibacillus residui DSM 22072T (FN293173)
S192T
S204T
S197T
S242T
Paenibacillus harenae DSM 16969T (AY839867)
Paenibacillus alkaliterrae DSM 17040T (AY960748)
S231T
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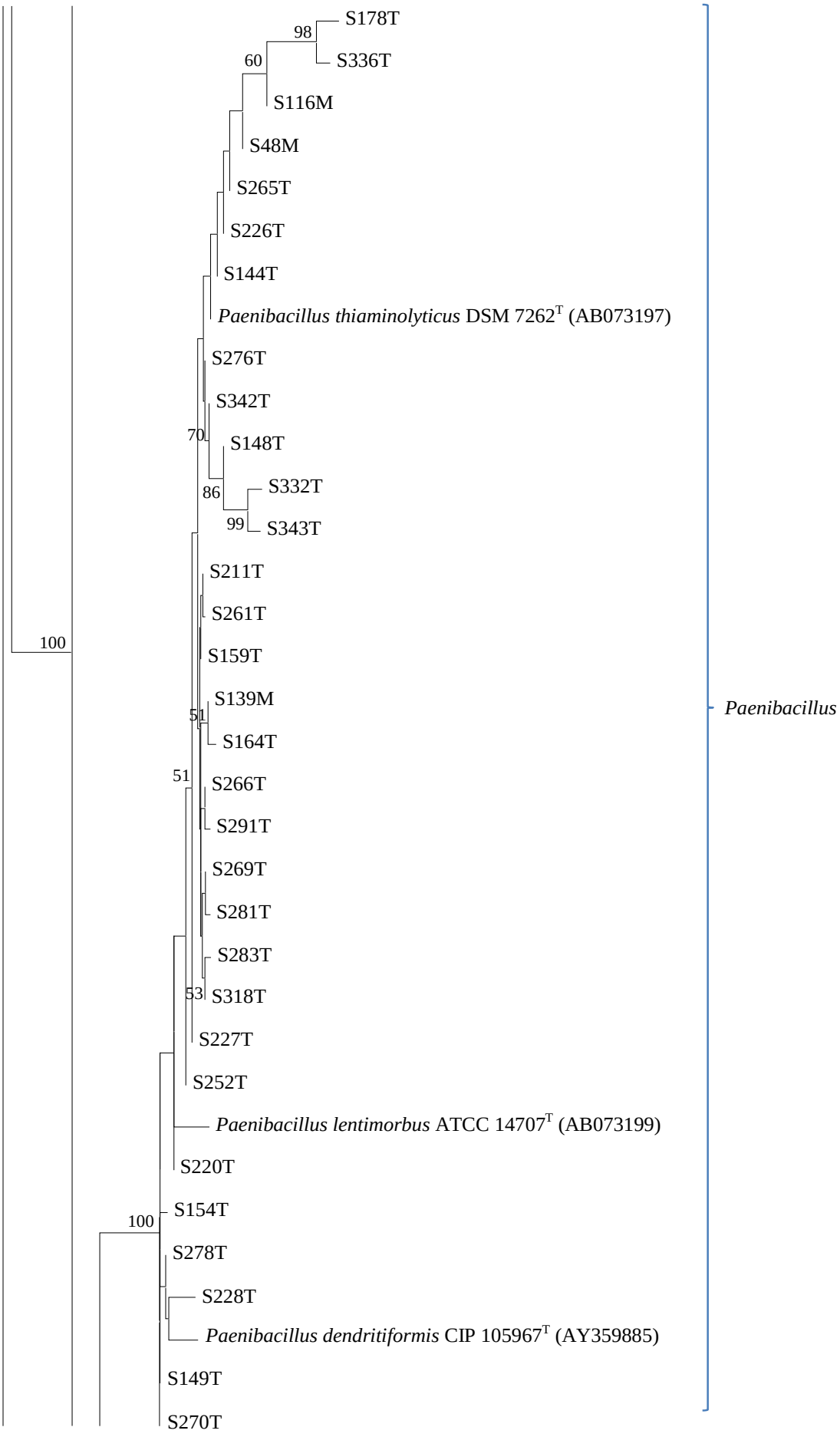
Paenibacillus daejeonensis DSM 15491T (AF290916)
S104M
S140M


S178T
98
S336T
60
S116M
S48M
S265T
S226T
S144T
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S342T
70
S148T
S332T
86
99
S343T
S211T
S261T
100
S159T
S139M
51
S164T
51
S266T
S291T
S269T
S281T
S283T
53
S318T
S227T
S252T
Paenibacillus lentimorbus ATCC 14707^{T} (AB073199)
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S149T
S270T
Paenibacillus

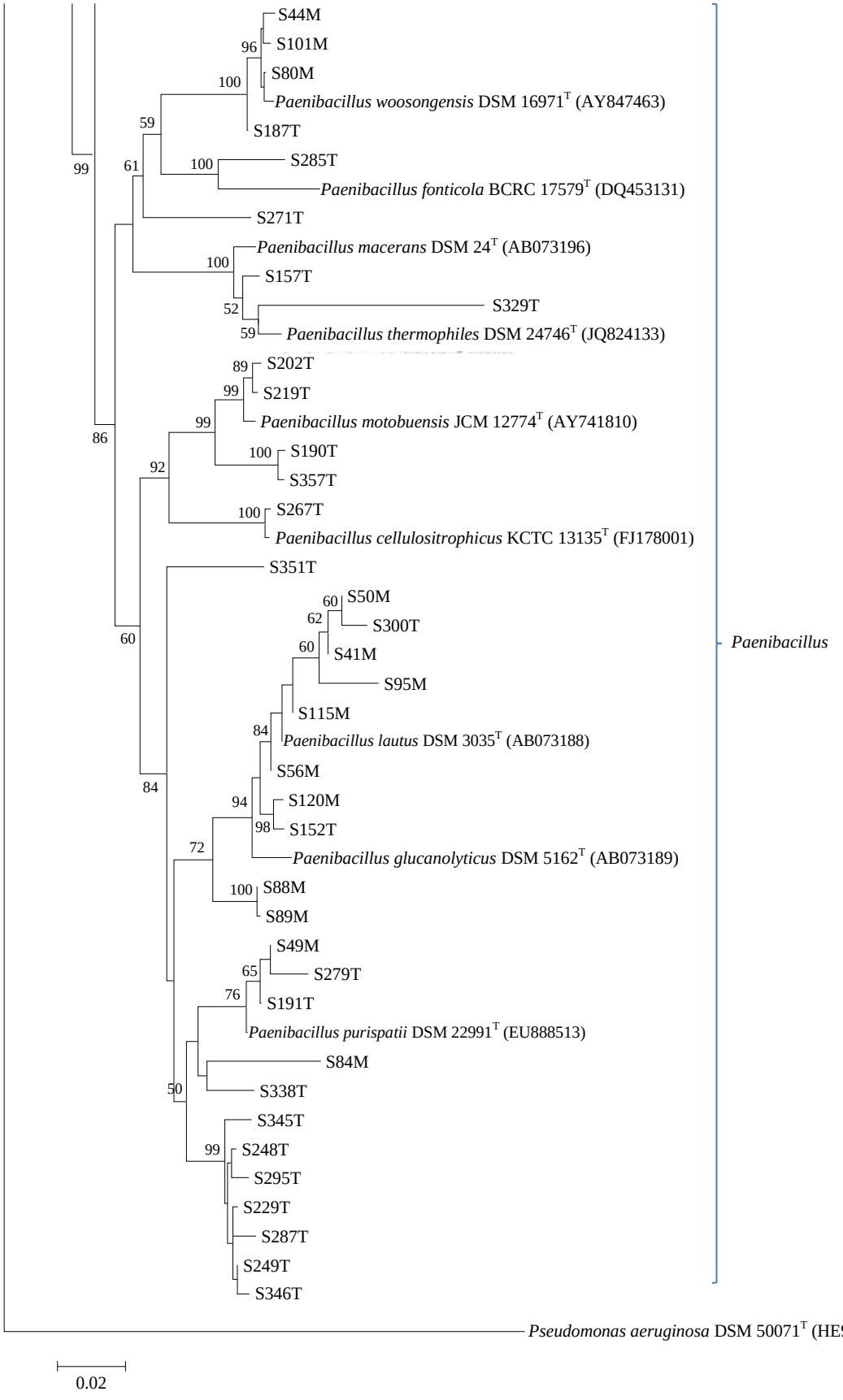


Figure 7.2: 16S rRNA gene sequence-based phylogenetic consensus tree constructed using the neighbour-joining algorithm, showing the position of Gram positive strains and their related genera. The sequence of *Pseudomonas aeruginosa* was used as an out-group. Bootstrap values of ≥ 50% based on 1000 replications are shown at the branching nodes. Bar, 0.02 substitutions per nucleotide position.

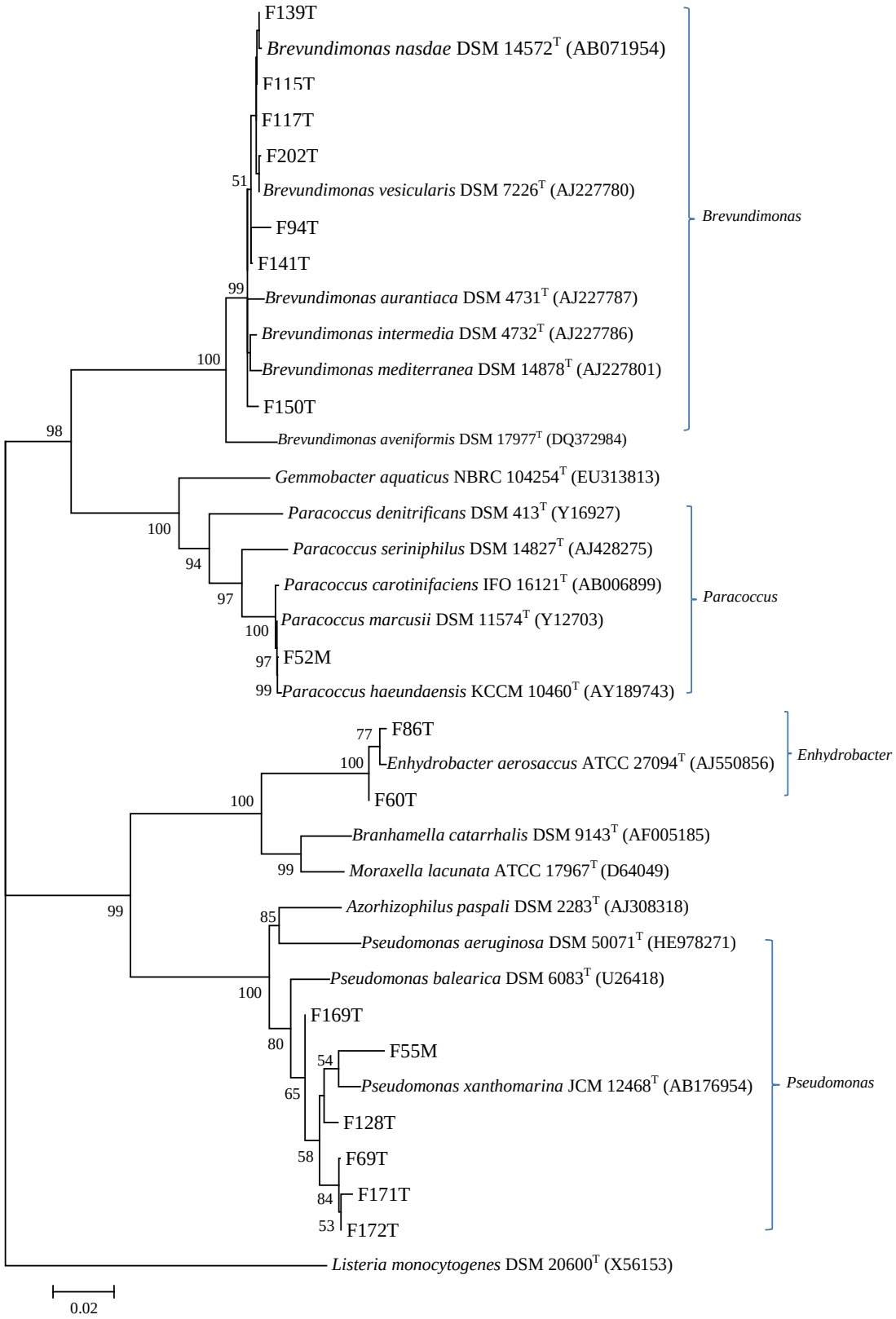
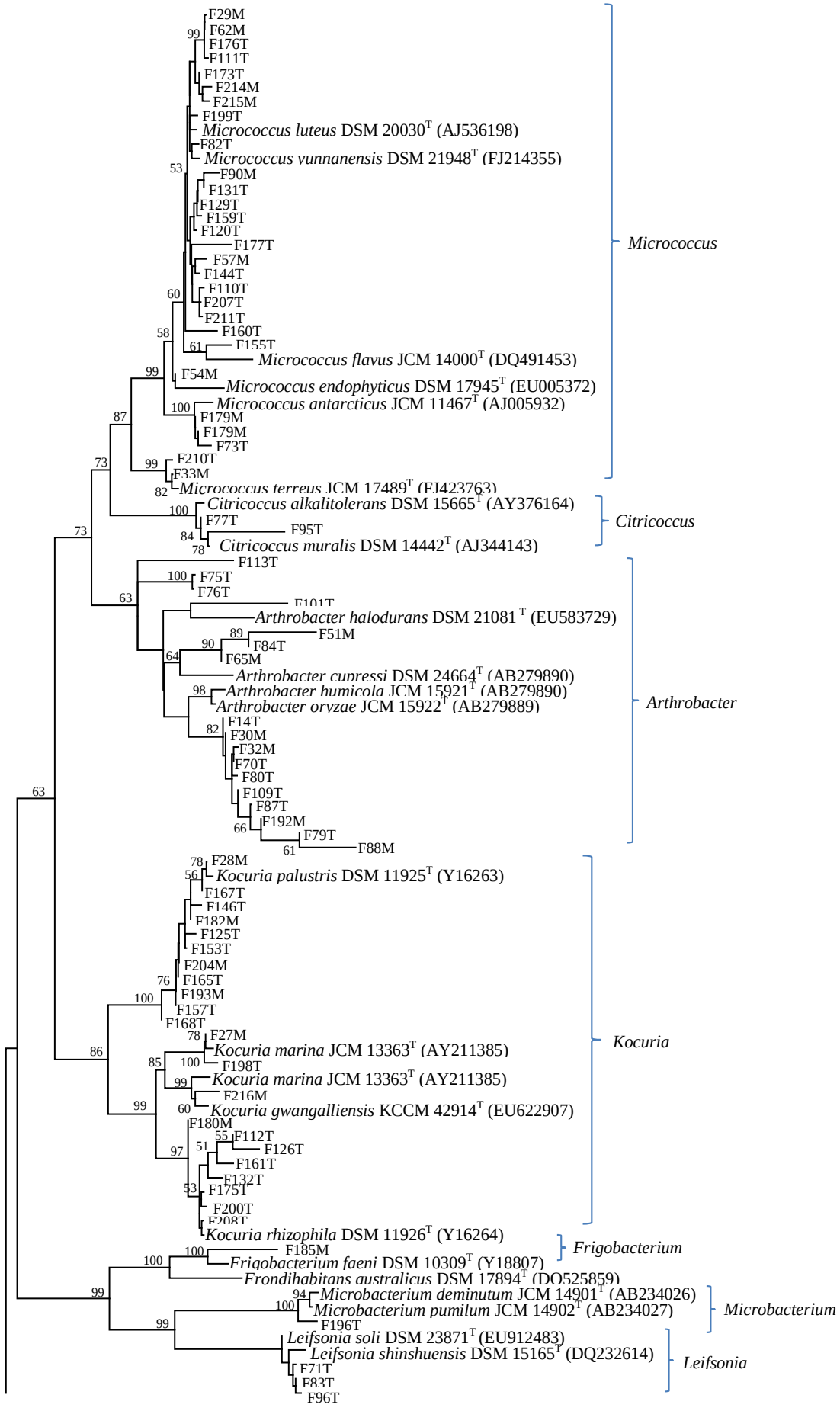


Figure 7.3: 16S rRNA gene sequence-based phylogenetic consensus tree constructed using the neighbour-joining algorithm, showing the position of Gram negative strains and their related genera from the representative of the suborder *Caulobacterales*, *Rhodobacterales* and *Pseudomonadales*. The sequence of *Listeria monocytogenes* was used as an out-group. Bootstrap values of ≥ 50% based on 1000 replications are shown at the branching nodes. Bar, 0.02 substitutions per nucleotide position.


F29M
F62M
F176T
F111T
F173T
F214M
F215M
F199T
Micrococcus luteus DSM 20030T (AJ536198)
F82T
Micrococcus yunnanensis DSM 21948T (FJ214355)
F90M
F131T
F129T
F159T
F120T
F177T
F57M
F144T
F110T
F207T
F211T
F160T
F155T
Micrococcus flavus JCM 14000T (DQ491453)
F54M
Micrococcus endophyticus DSM 17945T (EU005372)
Micrococcus antarcticus JCM 11467T (AJ005932)
F179M
F179M
F73T
F210T
F33M
Micrococcus terreus JCM 17489T (FJ423763)
Micrococcus
Citricoccus alkalitolerans DSM 15665T (AY376164)
F77T
F95T
Citricoccus muralis DSM 14442T (AJ344143)
Citricoccus
F113T
F75T
F76T
F101T
Arthrobacter halodurans DSM 21081T (EU583729)
F51M
F84T
F65M
Arthrobacter cupressi DSM 24664T (AB279890)
Arthrobacter humicola JCM 15921T (AB279890)
Arthrobacter orzyae JCM 15922T (AB279889)
F14T
F30M
F32M
F70T
F80T
F109T
F87T
F192M
F79T
F88M
Arthrobacter
F28M
Kocuria palustris DSM 11925T (Y16263)
F167T
F146T
F182M
F125T
F153T
F204M
F165T
F193M
F157T
F168T
F27M
Kocuria marina JCM 13363T (AY211385)
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Kocuria gwangalliensis KCCM 42914T (EU622907)
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F161T
F132T
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F200T
F208T
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Kocuria
F185M
Frigobacterium faeni DSM 10309T (Y18807)
Frondihabitans australicus DSM 17894T (DQ525859)
Frigobacterium
Microbacterium deminutum JCM 14901T (AB234026)
Microbacterium pumilum JCM 14902T (AB234027)
F196T
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Leifsonia

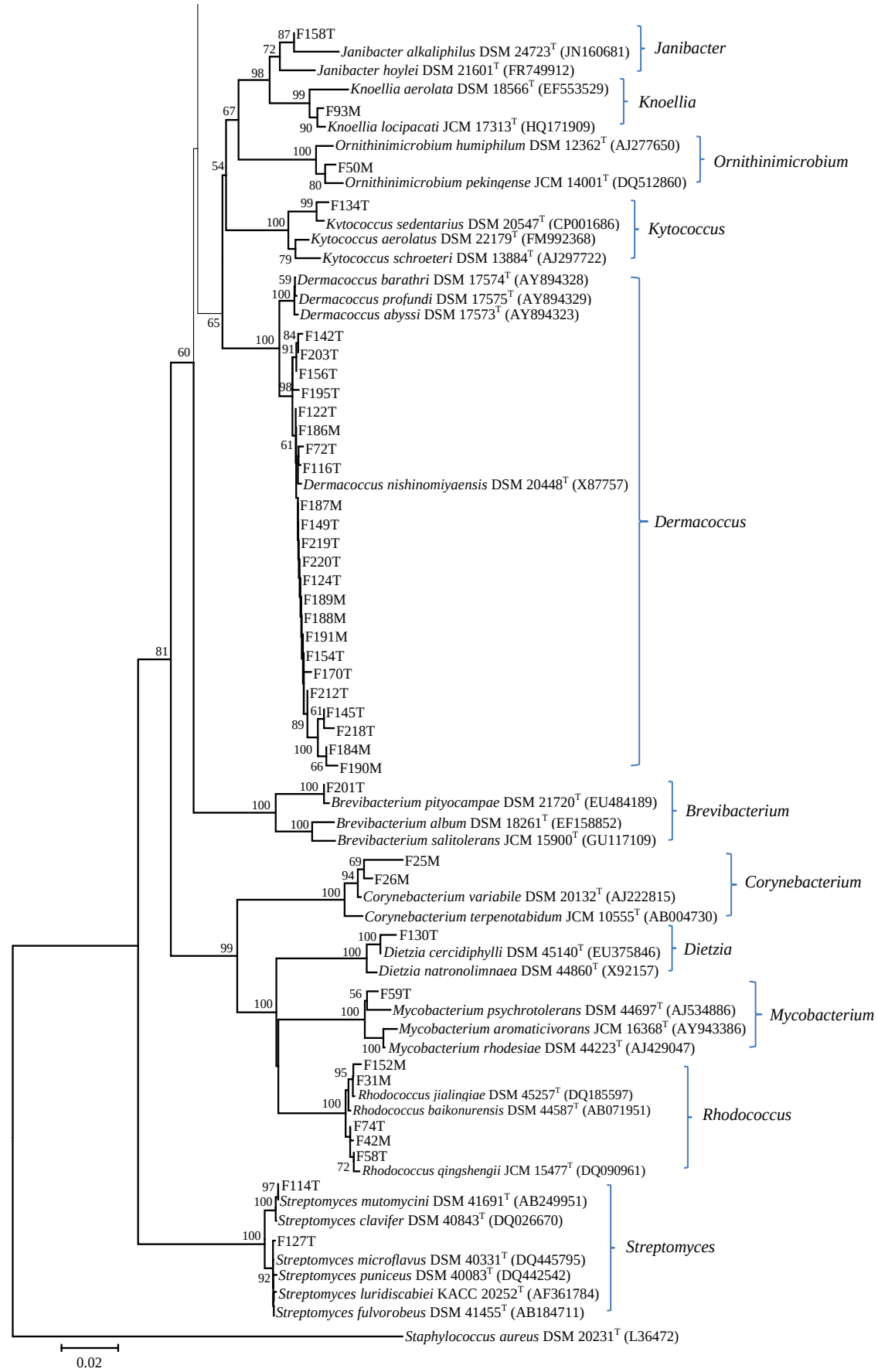


Figure 7.4: 16S rRNA gene sequence-based phylogenetic consensus tree constructed using the neighbour-joining algorithm, showing the position of *Actinobacteria* strains and their related genera. The sequence of *Staphylococcus aureus* was used as an out-group. Bootstrap values of ≥ 50% based on 1000 replications are shown at the branching nodes. Bar, 0.02 substitutions per nucleotide position.

7.3.3. Comparison of results between culture-dependent and molecular techniques

The culture-based findings were correlated to the results obtained from the PCR-DGGE and metagenomics molecular analyses (see Table 7.3 for data summary). In the freshwater sediments, culture-based technique revealed the presence of *Actinobacteria*, *Firmicutes* (Gram-positive) and Gram-negative bacteria of *Alphaproteobacteria* and *Gammaproteobacteria*. In contrast, the PCR-DGGE technique was able to detect the communities of *Betaproteobacteria* and *Archaea* but not the *Firmicutes*. However, in the brackish sediment, culture-dependent approach was unable to recover the Gram-negative bacteria. The metagenomics study based on 454 pyrosequencing analysis also showed the presence of *Firmicutes* as the predominating bacterial group and the minor groups of high GC *Actinobacteria* and *Alphaproteobacteria*.

Notably, the culture-based approach is capable of recovering a great deal of high GC Gram positive *Actinobacteria*, at levels significantly higher than those detected using PCR-DGGE technique in the freshwater sediments. On the contrary, the PCR-DGGE technique has shown to be better in detecting the Gram-negative bacteria. In the case of brackish sediment, the culture-dependent results have recovered a great number of representative *Firmicutes* (158). This is supported by the metagenomics approach which revealed 92% of predominating *Firmicutes*.

Clearly, the findings from molecular techniques have demonstrated some similarities with the data obtained from culture-based methods. As a whole, each technique has shown the ability to detect similar and slightly different bacterial groups which can altogether present an overview of the microbial diversity in Lake Suigetsu sediments.

Table 7.3: Comparison of taxa identified using culture-dependent and molecular techniques on Lake Suigetsu sediments

Freshwater sediment (6860 & 10911 BP)		**Brackish sediment (335 BP)**			
DGGE (Rr)	**Culture (Total Rep.)**	**Metagenomics (OTU %)**			**Culture (Total Rep.)**
Phylum	**Genus**	**Phylum**	**Family**	**Genus**	**Genus**
Acidobac. (45)	-	*Actinobac*.	*Micromonosporaceae*	*Actinoplanes* (0.03)	-
Actinobac. (21)	*Arthrobacter* (17)				*Janibacter* (1)
	Brevibacterium (1)	*Alphapro*.	*Bradyrhizobiaceae*	*Bradyrhizobium* (0.05)	-
	Citricoccus (2)	*Firmicutes*	*Alicyclobacillaceae*	*Alicyclobacillus* (57.74)	-
	Corynebacterium (2)			*Tumebacillus* (0.13)	-
	Dermacoccus (23)		*Bacillaceae*	*Bacillus* (15.42)	*Bacillus* (68)
	Dietzia (1)			*Geobacillus* (0.61)	-
	Frigobacterium (1)				*Oceanobacillus* (1)
	Janibacter (1)				*Paucisalibacillus* (1)
	Knoellia (1)				*Virgibacillus* (2)
	Kocuria (22)		*Catabacteriaceae*	*Catabacter* (0.03)	-
	Kytococcus (1)		*Clostridiaceae*	*Alkaliphilus* (0.16)	-
	Leifsonia (3)			*Caloramator* (0.11)	-
	Microbacterium (1)			*Clostridium* (13.38)	-
	Micrococcus (28)		*Gracilibacteraceae*	*Gracilibacter* (0.03)	-
	Mycobacterium (1)		*Lachnospiraceae*	*Cellulosilyticum* (0.13)	-
	Ornithinimicrobium (1)		*Paenibacillaceae*	*Ammoniphilus* (0.45)	-
	Rhodococcus (5)			*Brevibacillus* (0.55)	*Brevibacillus* (1)
	Streptomyces (2)			*Cohnella* (0.34)	-
Alphapro. (33)	*Brevundimonas* (7)			*Paenibacillus* (3.99)	*Paenibacillus* (84)
	Paracoccus (1)			*Thermobacillus* (0.13)	-
Betapro. (20)	-		*Peptococcaceae*	*Cryptanaerobacter* (0.11)	-
Gammapro. (18)	*Enhydrobacter* (2)			*Desulfitobacterium* (1.72)	-
	Pseudomonas (6)			*Desulfotomaculum* (0.82)	-
Firmicutes (0)	*Bacillus* (9)			*Pelotomaculum* (0.53)	-
	Caryophanon (1)			*Thermincola* (0.18)	-
	Jeotgalicoccus (1)		*Planococcaceae*	*Sporosarcina* (0.11)	*Sporosarcina* (1)
	Lysinibacillus (1)		*Ruminococcaceae*	*Acetivibrio* (0.45)	-
	Sporosarcina (2)			*Ethanoligenens* (0.11)	-
	Staphylococcus (22)			*Papillibacter* (0.05)	-
Archaea (40)	-			*Sporobacter* (0.21)	-
			Sporolactobacillaceae	*Tuberibacillus* (0.05)	-
			Streptococcaceae	*Streptococcus* (0.05)	-
			Syntrophomonadaceae	*Pelospora* (0.08)	-
				Syntrophomonas (0.42)	-

Freshwater sediment = B (N) 05/07; brackish sediment = A01; Rr indicates the number of DGGE bands/species; Total Rep. represents the total number of representative isolates recovered; OTU % indicates the percentage of taxa detected at family level. *Acidobac. = Acidobacteria*; *Actinobac*. = *Actinobacteria*; *Alphapro*. = *Alphaproteobacteria*; *Betapro*. = *Betaproteobacteria*; *Gammapro*. = *Gammaproteobacteria*

7.4. Discussion:

Conventionally, culture-based techniques were used in the studies of bacterial communities in which they were designed to exploit maximal recovery of different bacterial species from an environment by using various culture media (Hill *et al*., 2000). However these standard cultivation techniques often underestimate the diversity of bacterial communities from a complex environment (Vartoukian *et al*., 2010). The use of DDC in this study could improve the cultivation method by maximising the separation of bacteria from the sediments of Lake Suigetsu. DDC is a multi-step extraction process, first described by Hopkins *et al*. (1991) to separate and maximise the dispersion and disassociation of bacteria from soil aggregates of diverse soils. This multi-stage procedure has been demonstrated to be effective in extracting mycelial bacteria such as *Actinobacteria* and particularly streptomycetes from a range of soils (Gomes *et al*., 1999; Macnaughton and O'Donnell, 1994; Maldonado *et al*., 2005) compared to classical shaking methods (Atalan *et al*. 2000; Sembiring *et al*. 2000). Studies have also revealed that DDC technique enhanced the recovery of bacteria from three to nine-fold in comparison to those achieved through serial dilution method (Gomes *et al*., 1999; Zhao *et al*., 2009). Besides, various cultivable representatives of the genus *Streptomyces* have been successfully isolated through the successive stages of this procedure (Atalan *et al*., 2000; Hopkins *et al*., 1991; Sembiring *et al*., 2000). It was suggested that the combined physicochemical treatments of mild detergent (sodium cholate), buffering (Tris buffer), attenuated physical disruption (mild ultrasonication) and ionic shock (distilled water) in the process can enhance the breaking down of bacteria-particle associations (Atalan *et al*., 2000) and increase the number of viable counts over shaking. Moreover, the dispersion of soil by sonication not only can increase the mean viable count but also the variability between replicates (Janssen *et al*., 2002). Therefore, in this case, the DDC protocol could be considered as a more appropriate approach in separating the bacteria from the old and dense sediments as viable bacteria can be tightly bound to soil aggregates as deposition time increases.

7.4.1. Isolation of bacteria

Apart from DDC technique, laboratory cultivation conditions such as pH, temperature, media compositions and incubation period are also important in maximising the numbers of bacterial cells being recovered (Beales, 2004). In Lake

Suigetsu, the epilimnion temperature varies with seasons from 3°C during winter to 26°C in the summer (Kondo and Butani, 2007) while below the metalimnion, temperature remains constant at approximately 14°C throughout the year, irrespective of season (Kondo *et al*., 2006; Kondo and Butani, 2007). In order to recover bacteria from such environment, non-selective medium was employed. In fact, non-selective medium has been used for the studies of bacterial communities from soil (McCaig *et al*., 2001), deep-sea (Toffin *et al*., 2004), estuarine and freshwater sediments (Wuertz *et al*., 1991). It has been reported to yield a higher number of bacterial cells compared to selective media and it is suitable for the resuscitation of 'stressed' bacterial cells (Roszak and Colwell, 1987). In this study, TPA was chosen as the non-selective medium due to the fact that it is nutritious and supports a wide range of microorganisms, both the fastidious and non-fastidious bacteria from different environments (Wuertz *et al*., 1991), including soil (McCaig *et al*., 2001) and clinical specimens (Osterhout *et al*., 1991). This medium has also been reported to be able to recover bacterial cells from stress environments (Gurtler and Beuchat, 2005) and the addition of yeast extract was to enhance bacterial growth (Roszak and Colwell, 1987). Apart from the general-purpose medium of TPA that has been selected, MA medium was also utilised to mimic the saltwater conditions with the intention to recover bacteria from brackish sediments.

7.4.2. Bacterial taxa in freshwater and brackish sediments

By using TPA and MA media for cultivation at 25°C for a minimum of a week, ancient and viable bacteria from Lake Suigetsu sediment were able to be recovered. In general, there are two types of bacterial populations present in the marine and freshwater habitats, the oligotrophs and the copiotrophs. Oligotrophs are those that can survive in low nutrient environments while copiotrophs mainly grow in high nutrient concentration conditions that can still survive in low nutrient environments (Martin and MacLeod, 1984). However, in unpredictable natural environments, the conditions are often unfavourable for optimum microbial growth and reproduction. Lennon and Jones (2011) stated that when microorganisms encounter changes in abiotic factors from its surrounding such as nutrient availability, pH, temperature and osmotic pressure, dormancy will be triggered. Dormancy is a reversible phenotypic development of an organism and during this state, bacteria will lower their metabolic activity to slowly use up their energy reserves in order to survive the harsh environments (Lennon and Jones,

2011; Roszak and Colwell, 1987). Therefore, microorganisms that are capable of surviving in the anoxic and nutrient depleted sediments of Lake Suigetsu for a significant period of time could be those that may have persisted as vegetative cells or spores formers that become dormant when environmental conditions are harsh while resuscitating when conditions become favourable.

When comparing the number of representative isolates and the type of taxa being isolated from both water conditions, major differences were spotted in that the bacterial taxa isolated from freshwater sediments (BN05/07) were highly diverse compared to the brackish sediment (A01). Particularly, in the freshwater sediments, the *Actinobacteria* communities were vastly diverse which comprised of 19 distinct taxa while only one *Actinobacteria* taxon was found in brackish sediment. *Actinobacteria* are in fact common bacteria which not only can be found in soil (Janssen, 2006), but are also abundant in various freshwater habitats (Lindström and Leskinen, 2002; Lindström *et al*., 2005; Shade *et al*., 2007). Glöckner *et al*. (2000) also stated that more than 50% of the bacteria in lake surface waters are *Actinobacteria* and its abundance mainly decreases with oxygen concentrations. Although, many of the *Actinobacteria* members are capable of producing spores (Ventura *et al*., 2007), those that were recovered from the sediments of Lake Suigetsu were mainly non-spore-forming. Previously, these non-spore-formers have been isolated from the marine sediments (Gontang *et al*., 2007) and were found to survive ancient permafrost (Zhang *et al*., 2013). Their survival under extreme environments has been suggested to be due to adaptations connected to dormancy (Mulyukin *et al*., 2001) and tolerance to stresses in the environments (La Duc *et al*., 2007).

In addition, based on the amplified 4-kb bacterial DNA amplicons, *Arthrobacter*, with the capability of anaerobic metabolism, was found to be predominant in the sediments of ancient permafrost. Their cellular metabolic activity and DNA repair mechanisms were believed to be closely related to long term survival over time rather than dormancy (Johnson *et al*., 2007). Therefore, the highly diverse non-spore-forming freshwater *Actinobacteria* taxa recovered from the old sediments of Lake Suigetsu could possibly reflect adaptation, tolerance and the capability of DNA repair.

Similarly, 16S rRNA sequencing analysis based on cultured representative isolates also demonstrated that the bacterial taxa from freshwater sediments were largely different from those in brackish sediment. However, the number of representative isolates recovered from the freshwater sediments (BN 05/07) was just slightly higher than those isolated from the brackish sediment (A01). After the event of seawater incursion in Lake Suigetsu, diverse *Actinobacteria* taxa that were found present in the freshwater sediments were not detected in the brackish sediment. Instead, Gram positive bacterial taxa such as *Bacillus* and *Paenibacillus* became predominant in this sediment. Such occurrence may suggest that most of the *Actinobacteria* taxa from the freshwater sediments are salt-intolerant in comparison to *Bacillus* and *Paenibacillus*. In general, *Bacillus* and *Paenibacillus* from the phylum *Firmicutes* are halotolerant bacteria that are frequently detected in low- and high-salinity sediments (Benlloch *et al*., 2002; Bowman *et al*., 2000; Li *et al*., 1999b).

In contrast, *Actinobacteria* have varying degrees of salt tolerance that is largely depended on the habitats they originate from (Jensen *et al*., 2005). Goodfellow and Williams (1983) reported that *Actinobacteria* from the marine habitats are usually more salt tolerant compared to the terrestrial ones, whereas those among sand dunes are more tolerant than those from the seawater. Therefore, species replacement, cell inactivation or death could occur with changes in salt concentrations (del Giorgio and Bouvier, 2002; Wu *et al*. 2006). Besides, the difference in bacterial taxa between the two conditions could be that the taxa originally adapted to freshwater conditions were replaced by other taxa which can better adapt to the current salinity conditions (Hart *et al*., 1991; Nielsen *et al*., 2003). In this case, *Bacillus*, *Sporosarcina* (from the *Firmicutes* phylum) and *Janibacter* could possibly possess broad ecophysiological capabilities as they were both detected in both sediment conditions (Wu *et al*., 2006). *Firmicutes* as discussed in Chapter 4, are freshwater and halotolerant organisms that are also predominate in low-salinity sediments, including the deep-sea sediments (Li *et al*., 1999b) and meromictic lakes of marine salinity from Eastern Antarctic (Bowman *et al*., 2000). The high GC Gram positive *Janibacter* on the other hand are bacteria that are capable of changing morphology during growth (Martin *et al*., 1997). The members of *Janibacter* have been detected not only in the natural environments (Kageyama *et al*., 2007) that included the sea sediment (Hamada *et al*., 2013) but also in clinical specimen (Loubinoux *et al*., 2005).

7.4.3. Comparison of microbial diversity between culture-dependent and molecular approaches

Knowing the limitations of culture-based approaches, this single method is clearly inappropriate to be directly performed on the whole microbial diversity analyses (Tamaki *et al*., 2005). Our data demonstrated that culture-dependent method can recover a higher number of high GC Gram positive *Actinobacteria* from the freshwater sediments and a greater number of Gram positive bacteria (i.e. *Bacillus* and *Paenibacillus*) from the brackish sediment than PCR-DGGE technique. In contrast, PCR-DGGE has shown to better recover Gram negative bacteria (i.e. *Proteobacteria*) as well as *Acidobacteria* which were not detected by the culture-based technique at all. Indeed, our findings are comparable to previous studies which have shown that culture-dependent techniques can reveal a wide diversity of high GC Gram-positive bacteria at a remarkably high percentage (Smit *et al*., 2001). Molecular approaches on the other hand can detect better profiles of difficult-to-culture yet widespread genera such as *Acidobacteria* in soil despite the use of improved cultivation methods (Barns *et al*., 1999; Janssen *et al*., 2002; Kielak *et al*., 2009; Kleinsteuber *et al*., 2008; Sait *et al*., 2006). Even with improved cultivation methods, new isolates that have been recovered from soil were affiliated with *Acidobacteria* subdivisions 1-4 whereas the diversity detected using molecular approach was largely represented by the 6 subdivisions in clone libraries (Janssen, 2006). Besides, the PCR amplification of high GC templates has also been reported to be difficult in comparison to non-GC rich targets (McDowell *et al*., 1998). According to Mamedov *et al*. (2008), most of the housekeeping and tissue-specific genes (~40%) contain high GC sequences in their promoter regions, hence DNA is less amenable to amplification.

Apart from this, in the brackish sediment, the metagenomics study based on 454 pyrosequencing revealed distinctive bacterial taxa from culture-dependent method. 454 pyrosequencing not only detected taxa that are environmentally related, the direct sequencing of environmental DNA evidently disclosed a higher number of taxa at genus level (31) than the culture-based technique which recovered only 7 genera, although some of the taxa from metagenomics study are comparable to the culture data. This is largely due to the limitations of culture-dependent approach as in general, the choice of the growing medium can significantly influence the colony formed (Johnsen and Nielsen, 1999), however there is not yet a single medium which can fully support

the growth of all bacteria from a mixed and complex community (Bussmann *et al*., 2001). The differences in bacterial diversity shown between different techniques could be mainly due to the limitations of cultivation media, which would not perfectly suit all types of bacteria in Lake Suigetsu sediments. Nonetheless, not all the isolates that were recovered on the isolation media were able to be subcultured, particularly the comparatively small sized colonies. As known, natural environment is not an optimal environment for the growth of all bacteria and such small sized bacteria that were found grown on the culture media could be the oligotrophs (Roszak and Colwell, 1987). According to some studies, the transfer of oligotrophs from a nutrient deprivation environment to a richer condition will result in the growth of small bacteria in which they are in the starvation mode and unable to endure the earlier rate of metabolism (Velimirov, 2001), therefore fail to multiply. Ultramicrobacteria or 'dwarf' cells are another types of small bacteria cells which commonly inhabit aquatic environments. They are also 'non-culturable' as they cannot form colonies on agar medium at all (Roszak and Colwell, 1987; Velimirov, 2001). In this case, molecular techniques that do not require cultivation will be more advantageous for the detection of these oligotrophs.

Besides media composition, the incubation period is also one of the major factors that can lead to selected bacteria being cultivated (Goodfellow and Williams, 1983). The discrepancy is essentially due to the interdependency of different organisms upon each other (i.e. bacteria with symbiotic lifestyle), to the inability to create in pure culture the environmental conditions faced by microorganisms in the soil environment, and to the fact that some microbial species are cultivable only under certain physiological conditions (Muyzer and Smalla, 1998; Heuer *et al*., 2001). In addition, Roszak and Colwell (1987) stated that the appropriate temperature is essential and temperature significantly greater than 20°C is lethal for aquatic bacteria or bacteria that have adapted to the aquatic environment. In this study, an incubation temperature of 25°C was set to target not only the freshwater bacteria but also the common soil *Actinobacteria*. This is because the temperature usually used for a wider coverage of *Actinobacteria* genera is at 25°C to 30°C (Goodfellow and Williams, 1983). Therefore, incubation temperature at 25°C can potentially inactivate bacteria that cannot grow at temperature higher than 20°C.

Moreover, Gram-negative *Betaproteobacteria*, *Acidobacteria* and *Archaea* that were detected using PCR-DGGE from the freshwater sediments, were not recovered

using the culture-dependant approach. Former studies have reported that *Betaproteobacteria* are the most extensively studied bacterial group in freshwater lakes and in general, they inhabit the upper waters of the lakes, are nutrient loving and are fast growing, thereby amenable to culturing (Šimek *et al*., 2005; 2006). The detection of this phylum in the sediments by PCR-DGGE could be that they were the metabolically non-active bacterial cells from the upper layer of lake waters which have been deposited in the lake sediments, thereby could not be recovered on the culture media; likewise, the *Gammaproteobacteria*. Additionally, the absence of *Acidobacteria* could be mainly due to the unsuitable pH level of the media prepared in this study as they were designed to generally suit a wider range of bacterial growth. In fact, *Acidobacteria* are ubiquitous and abundant members of soil bacterial communities. However, the ecological characteristics of this dominant phylum in natural environments are still vague owing to the members of this phylum being difficult to culture (Jones *et al*., 2009; Ward *et al*., 2009). Studies have shown that the *Acidobacteria* members from subdivisions 1, 2, 3, and 4 do not grow on standard media and they are very slow-growing (from several days to weeks) requiring complex and low-nutrient media for growth (Davis *et al*., 2005; Eichorst *et al*., 2007; Joseph *et al*., 2003). Similarly, a study based on quantitative PCR found that the abundance of *Acidobacteria* often associated with low soil carbon availability in older soils (Fierer *et al*., 2007; Nemergut *et al*., 2007; Tarlera *et al*., 2008). Other molecular studies also found that *Acidobacteria* are at highest abundances in environments with the lowest pH (Fierer *et al*., 2007; Mannisto *et al*., 2007; Lauber *et al*., 2008). Therefore, these findings suggest that *Acidobacteria* are slow-growing oligotrophs and nutritious TPA and MA media with neutral pH are not suitable for their growth. Likewise, the absence of *Archaea* community from culture media would be that the temperature and media used were inappropriate as they required a more complex enrichment media at temperature of 42°C (Ochsenreiter *et al*., 2002).

On the other hand, metagenomics analysis revealed a great deal of mostly Gram positive bacteria compared with culture-based analysis. Through the metagenomics study, *Alicyclobacillus* (57.74%) was shown to be the predominant taxa in the brackish sediment followed by *Bacillus* (15.42%), *Clostridium* (13.38%) and *Paenibacillus* (3.99%) while culture-dependent analysis revealed a high number of members belonging to the genera *Paenibacillus* (86) and *Bacillus* (65). Although both techniques also demonstrated that *Fimicutes* are the predominant taxa in the brackish sediment, in general, metagenomics analysis was able to reveal higher numbers of *Firmicutes* taxa

(29) compared to culture-dependent analysis (6). For metagenomics study, 454 pyrosequencing was applied in which fossil DNA of an entire microbial community was initially extracted from the sediment, followed by the amplification of a particular target region flanked by conserved primers by PCR before sequencing (Balzer *et al*., 2010). During this process, an amplicon dataset will be generated and the variation in sequences between each read stems from a homologous region, reflecting the phylogenetic diversity in the community (Quince *et al*., 2009).

Interestingly, in a study by Lu *et al*. (2010), *Firmicutes* strains related to *Bacillus* and *Alicyclobacillus* have been isolated in deep and slightly acidic sediment zones with pH 4 to 5. In particular, *Alicyclobacillus* have been identified as Fe (III)-reducing bacteria responsible for iron cycling process which have acidophilic characteristic that can inhabit a wide ecological niche with maximum pH growth range from 1.5 to 6 (Lu *et al*., 2010). Besides, two members from genus *Alicyclobacillus*, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidiphilus*, been detected in several spoiled commercial pasteurised fruit juices and they have been identified as thermo-acidophilic, non-pathogenic and spore-forming bacteria. They are resistant to the pasteurisation treatment conditions normally applied to acidic fruit products and germinate and grow causing spoilage (Matsubara *et al*., 2002; Pettipher *et al*., 1997). Hence, the primary conditions for these thermo-acidophilic bacteria to grow will require high growth temperature at 45°C and an acidic environment (Matsubara *et al*., 2002).

In addition, members of *Clostridium* are largely found in soil, sewage and aquatic habitats, including both the freshwater and sea sediments (Dobbin *et al*., 1999; Emerson and Cabelli, 1982; Hielm *et al*., 1998). *Clostridium* species have been detected more abundantly in sea samples than freshwater (Hielm *et al*., 1998). Other members such as *Clostridium beijerincikii*, have been found to be capable of reducing Fe (III) in freshwater sediment, similar to *Alicyclobacillus*. *Clostridium* is a genus of spore-forming, obligate anaerobe and heterotrophic bacteria that require strict anaerobic conditions (Dehning and Schink, 1989; Molongoski and Klug, 1976). Light would therefore limit bacterial productivity and similarly, minute amounts of oxygen could be very toxic to the bacteria. In this case, metagenomics study is more useful in detecting anaerobes as in this study, the anaerobic cultivation was unsuccessful. The unsuccessful attempts could be due to the fact that isolates were very sensitive to oxygen and minute

amounts of oxygen could be very poisonous for them, as a result, purification and storage of these strains will be very difficult.

On the whole, bacterial species that exist in higher total biomass and metabolic activity in the environment may have more ecological significance than those that exist in small numbers (Bakken and Olsen, 1987) given that they could serve as indicators responsive to physical, chemical, and biological changes in the natural environments (Ellis *et al*., 2003). Despite the limitations of culture-based methods, these techniques need improvements not only to better reveal the conditions of microbial life in the ecosystems but also can increase the possibility of cultivating new as yet uncultured bacteria (Bussmann *et al*., 2001), given that there is growing interest in the studies of microbial ecology and functions (Lennon and Jones, 2011). Therefore, various methods that have been suggested to be capable of improving the total number of cultivated cells and resuscitate stressed bacterial cells include, the use of diluted media (Rappé *et al*., 2002; Zengler *et al*., 2002; Roszak and Colwell, 1987), low nutrients concentrations (i.e. peptone/yeast extract) (Velimirov *et al*., 2011), suitable metabolites (0.1% of pyruvate, acetate or oxalacetic acid) (Heinmets, 1953), extended incubation period (Goodfellow and Williams, 1983; Song *et al*., 2009; Vartoukian *et al*., 2010), dilution-to-extinction (dilute samples before cultivation) (Song *et al*., 2009) as well as mimic the natural environment conditions where the bacteria originate (Rappé *et al*., 2002; Song *et al*., 2009).

Metagenomics studies in this case can be more useful in the sense that it involves direct sequencing of environmental DNA which can be more accurate than PCR-DGGE. PCR-DGGE can result in a less refined phylogenetic interpretation (Díez *et al*., 2001) due to the fact that DGGE bands sequences are very short and typically only 500 bp of sequence can be analysed (Muyzer, 1999). Although culture-based techniques are time-consuming and laborious, they are less expensive and readily available in comparison to molecular techniques, i.e. pyrosequencing, an expensive technique that enables rapid characterisation of microbial communities at greater sequence depth (Sogin *et al*., 2006).

7.5. Conclusions:

Overall, this study shows that ancient bacteria from old sediments are ‘culturable’ and viable. Molecular approaches have been shown to address a larger proportion of the bacterial community compared to culture-based technique which is only limited to ‘culturable’ bacteria as well as bacteria that could respond quickly to given nutrients and concentrations (McCaig *et al*., 2001). This study also clearly shows that each technique has different strengths in recovering different bacterial taxa which can complement one another whilst offering a more complete profile of microbial diversity in Lake Suigetsu sediments. Further work is evidently needed, such as the excision of bands from the PCR-DGGE analysis, for sequencing or cloning so that the comparison between techniques can be more comparable. According to Vartoukian *et al*. (2010) this combined use of techniques will be the future direction in further improving the range of bacterial life that can be cultured.

Chapter 8 Classification of novel *Actinobacteria* from the genera of *Dermacoccus*, *Dietzia*, *Leifsonia* and *Rhodococcus*.

8.1. Introduction

In addition to their presence in terrestrial soils and marine environments, one of the most abundantly found and ubiquitous freshwater bacteria known, is the *Actinobacteria* (Allgaier and Grossart, 2006; Goodfellow and Williams, 1983; Maldonado *et al*., 2005; Newton *et al*., 2011). Their ubiquity may in part be due to a small cell size that can enable them to resist grazing from predation in aquatic environments (Newton *et al*., 2011). It has also been postulated that the abundance of this phylum, usually at the upper water surface of the lake, is due to their capability to resist UV stress (Warnecke *et al*., 2005). In addition, numerous members of the *Actinobacteria* taxon are capable of producing spores which allow them to withstand harsh and extreme environments (Ventura *et al*., 2007). A recent study by Antibus *et al*. (2012) has reported that with their ability to undergo dormancy and persist in cold and dry climates, metabolically active *Actinobacteria* have been found surviving the Antarctic environment for thousands of years.

Goodfellow and Fiedler (2010) stated that the genus *Streptomyces* in particular is the richest source of natural products as it accounts for 80% of the total known bioactive secondary metabolites (7,600 compounds). Therefore, there is a need to continuously search for new *Actinobacteria*. Similarly, new drugs especially antibiotics are also urgently needed in order to encounter drug-resistant pathogens and to treat life-threatening diseases like cancer (Newman and Cragg, 2007; Olano *et al*., 2009). In fact, the antibiotics synthesised from microorganisms are known to be natural and the most promising source for drugs in comparison to chemically designed drugs (Bull and Stach, 2007).

Besides, rare biospheres have been suggested to be possible habitats for the discovery of new bacterial species as these environments are ancient and could contain rich sources for genomic innovation (Sogin *et al*., 2006). Besides, through the use of improved culture-dependent techniques, habitats which are poorly studied are also

potential opportunities for the discovery of novel *Actinobacteria* (Goodfellow and Fiedler, 2010; Warnecke *et al*., 2004). Likewise, in the old freshwater sedimentary records of Lake Suigetsu, several genera of *Actinobacteria* were discovered and, among the isolates, new species were able to be identified. There were total of 18 distinctive *Actinobacteria* representative isolates identified and four among these representative isolates were chosen for further characterisation and description, including strains from the genera of *Dermacoccus*, *Dietzia*, *Leifsonia* and *Rhodoccocus*. The four members were chosen as they had shown to be the presumptive novel isolates based on phylogenetic analysis of the 16S rRNA gene. Additionally, these members from *Dermacoccus, Dietzia*, *Leifsonia* and *Rhodoccocus* genera were also selected for the ease of testing as the range of known type strains for comparison are not as numerous as for other genera.

At present, there are four species in the genus of *Dermacoccus*, including *Dermacoccus nishinomiyaensis* DSM 20448^T (previously classified as *Micrococcus nishinomiyaensis*; Stackebrandt *et al*., 1995), *Dermacoccus abyssi* DSM 17573^T (a piezotolerant actinomycete) (Pathom-aree *et al.*, 2006a), *Dermacoccus barathri* DSM 17574^T and *Dermacoccus profundi* DSM 17575^T (Pathom-aree *et al*., 2006b) (www.bacterio.net/d/dermacoccus.html). Previously, the members of this genus have been isolated from terrestrial habitats, cured meat product and human skin (Cordero and Zumalacárregui, 2000; de la Rosa *et al*., 1990; Papamanoli *et al*., 2002). In addition, these members have also been isolated from the sea sediment and deep-sea mud (Pathom-aree *et al.* 2006b).

In this study, the *Dermacoccus* isolates were recovered from the freshwater sedimentary records of Lake Suigetsu. *Dermacoccus* cells are Gram-positive, aerobic, non-spore-forming, non-acid–alcohol-fast, non-motile, formed irregular clusters of coccoid cells with diameter ranging from 0.8 to 1.5 mm. They grow well on trypticase soy agar but not on inorganic nitrogen agar. They are chemoorganotrophic, mesophilic but not halophilic (Stackebrandt *et al*., 1995). Their peptidoglycan types that have been found include type L-Ala–D-Glu, D-Ala–D-Glu, L-Lys–L-Ser and D-Ala–L-Lys–L-Ser (Panthom-aree *et al*., 2006a; 2006b). The chemical properties of *Dermacoccus* species are consistent with one another such as the lack of mycolic acids, contain dihydrogenated menaquinones (Panthom-aree *et al*., 2006a; 2006b; Stackebrandt *et al*., 1995) and with an A4*a* peptidoglycan type *sensu* (Schleifer and Kandler, 1972).

However, the fatty acid methyl esters profile varies between members and is largely dependent on growth conditions (Panthom-aree *et al*., 2006a).

The genus *Dietzia* proposed by Rainey *et al*. (1995) for a species that was formerly *Rhodococcus maris* (Nesterenko *et al*., 1982). Currently, the genus encompasses 13 species with validly published names (www.bacterio.net/d/dietzia.html), including *Dietzia maris* (the type species) (Rainey *et al*., 1995), *Dietzia natronolimnaea* (Duckworth *et al*., 1998); *Dietzia psychralcaliphila* (Yumoto *et al*., 2002); *Dietzia kunjamensis* (Mayilraj *et al*., 2006a); *Dietzia cinnamea* (Yassin *et al*., 2006); *Dietzia schimae* and *Dietzia cerdiciphylli* (Li *et al*., 2008); *Dietzia papillomatosis* (Jones *et al*., 2008); *Dietzia lutea* (Li *et al*., 2009); *Dietzia timorensis* (Yamamura *et al*., 2010); *Dietzia aerolata* (Kämpfer *et al*., 2010); and *Dietzia alimentaria* (Kim *et al*., 2011); and *Dietzia aurantiaca* (Kämpfer *et al*., 2012).

The members of this genus have been isolated from various soils, marine environments (Colquhoun *et al*., 1998; Duckworth *et al*., 1998; Mayilraj *et al*., 2006a; Nesterenko *et al*., 1982; Rainey *et al*., 1995; Takami *et al*., 1997) and clinical materials (Jones *et al*., 2008; Bemer-Melchior *et al*., 1999; Pidoux *et al*., 2001; Yassin *et al*., 2006). Interestingly, *Dietzia natronolimnaea* was isolated from soda lake environment, unlike the *Dietzia* isolate that was recovered from meromictic Lake Suigetsu. *Dietzia* cells are in general aerobic, Gram-positive, non-motile, non-spore-forming, coccoid-like cells that develop into short rods, chemoorganotrophic and with optimum growth at 28°C (Li *et al*., 2009; Rainey *et al*., 1995; Yamamura *et al*., 2010). The members of the genus *Dietzia* share similar chemical properties, such as the presence of mycolic acids and they possess dihydrogenated menaquinone MK-8(H_2) as the predominant menaquinone (Jones *et al*., 2008; Kämpfer *et al*., 2010; Li *et al*., 2008; Rainey *et al*., 1995; Yamamura *et al*., 2010).

The genus *Leifsonia* is a member of the family of *Microbacteriaceae*, in the order of *Actinomycetales* as described by Suzuki *et al*. (1999) and Evtushenko *et al*. (2000). At present, the genus contains 17 recognised species (www.bacterio.net/l/leifsonia.html) with *Leifsonia aquatica* DSM 20146^T as the type strain; members of the genus *Leifsonia*, have been isolated from various sources including soil (Dastager *et al*., 2008; 2009), roots (Evtushenko *et al*., 2000; Qiu *et al*., 2007), plants (Davis *et al*., 1984), distilled water (Leifson, 1962) as well as various

glacial environments (Pindi *et al*., 2009; Reddy *et al*., 2003; 2008). *Leifsonia* isolates recovered from this study represent the first members isolated from lake environments. The general features of the members of the genus *Leifsonia* comprise of them being obligate aerobes, Gram-positive, non-spore-forming cells that form irregular rods or filamentous structures. They are mesophilic, with optimum growth at 24 to 28°C. *Leifsonia* possess 2,4-diaminobutyric acid in the cell wall peptidoglycan, MK-11 as the major menaquinone, but do not contain mycolic acids (An *et al*., 2009; Evtushenko *et al*., 2000; Pindi *et al*., 2009).

The genus *Rhodococcus* was created by Zopf (1891) to describe the species of red pigment (lipochrome)-producing bacteria which belongs to the family of *Nocardiaceae* in the phylum of *Actinobacteria*. However, the genus name *Rhodococcus* has recently been found to have been already applied to an algal taxon by Hansgirg in 1884 (Tindall, 2014). So, despite the fact that the name *Rhodococcus* (Zopf, 1891) was included on the Approved Lists of Bacterial Names, it has been recently classified as illegitimate (Tindall, 2014). Currently, there are 38 recognised species in this genus (www.bacterio.net/qr/rhodococcus.html), with *Rhodococcus rhodochrous* DSM 43241^{T} as the type strain. Members of this genus have been isolated from marine sediments but have not been detected from lake sediment. Species of this genus have been detected in a wide variety of environments such as wastewater, cold desert and mainly in soils habitats (Goodfellow *et al*., 2002; Li *et al*., 2007; 2011; Mayilraj *et al*., 2006b; Kämpfer *et al*., 2014, Wang *et al*., 2008; Yoon *et al*., 2000a, 2000b). In addition, members of this genus also have environmental and biotechnological importance due to their extensive catabolic diversity and enzymatic activities (Warhurst and Fewson, 1994; Bell *et al*., 1998).

The members of this genus contain strains of industrial importance as many of them have been used for amino acid production, transformation of steroids and able to degrade a range of xenobiotics such as the toxic aromatic compounds (Peczynska-Czoch and Mordarski, 1984; Yoon *et al*., 2000a), including chlorinated phenols (Briglia *et al*., 1996), dinitrophenol (Lenke *et al*., 1992) and naphthalene (Grund *et al*., 1992). The genus *Rhodococcus* is also a very important taxon in terms of bioremediation (Yoon *et al*., 2000b). For instance, they have been found to be capable of degrading pyridine, the harmful and potential carcinogen aromatic compounds that are associated with the use of herbicides and insecticides (Jori *et al*., 1983; Leenheer and Stuber,

1981). In general, *Rhodococcus* cells are aerobic, Gram-positive, non-acid-fast, non-motile, formed rods, filaments or show elementary branching at early growth phase and they are mostly cocci in the stationary phase (Matsuyama *et al*., 2003; Yoon *et al*., 2000a). *Rhodococcus* members contain mycolic acids and their predominant isoprenoid quinone is the dihydrogenated menaquinone of MK-8(H_2) (Goodfellow *et al*., 2002; Matsuyama *et al*., 2003; Zhang *et al*., 2002).

In this study, five *Dermacoccus* isolates, one strain each from the genera, *Dietzia* and *Leifsonia* with two from the genus *Rhodococcus* were selected to undergo a polyphasic taxonomic analysis alongside their neighbouring type strains, including establishing the chemotaxonomic, genotypic and phenotypic differential characteristics, in order to determine the taxonomic status of these isolates in comparison to their closest type strains.

8.2. Experimental strategy:

In order to further characterise the potentially new *Actinobacteria* isolates, a series of phenotypic analyses including temperature, pH and salt tolerance tests was carried out. Test strains and their closely related type strains were subjected to acid production analysis based on 27 different carbon sources, 9 biochemical analyses and 22 degradation tests. The utilisations of carbon and energy sources (at 1%, 0.1%) and carbon and nitrogen sources (at 0.1%) were also analysed using Stevenson's medium. The enzymatic activities of the test strains were tested using API ZYM strips (bioMérieux). In addition, 1D-TLC was employed to analyse the mycolic acids of test strains while 2D-TLC was used to detect the polar lipids. The fatty acids of test strains were also determined using GC-MS.

8.3. Results:

8.3.1. Phylogenetic analysis

The evolutionary position of the five *Dermacoccus*, one *Dietzia*, one *Leifsonia* and two *Rhodococcus* isolates and their related species with the use of 16S rRNA gene sequence analysis was identified and confirmed using three different tree-making algorithms including neighbour-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) with 1000-

replicates to establish bootstrap confidence values calculated based on neighbour-joining analysis. Strains F124T^T, F142T^T, F156T^T, F218T^T and F195T^T were recovered within the genus *Dermacoccus* (Figure 8.1). This association was supported by all the tree-making algorithms with a 100% bootstrap value in the neighbour-joining analysis. All these strains shared the highest 16S rRNA gene sequence similarities with *D. nishinomiyaensis* DSM 20448^T at 99.79%, 99.35%, 99.50%, 99.09% and 99.71%, respectively with nucleotide differences ranging from 3 to 13 while the similarities between each strain were ranging from 99.07% to 99.86%, with 2 to 13 nucleotide differences (Appendix 13).

Strain F130T^T that belongs to *Dietzia* clade was found to be most similar to *D. cerdiciphylli* DSM 45140^T (99.70%) and *D. natronolimnaea* DSM 44198^T (99.63%) which also corresponds to 4 and 5 nucleotide differences (Appendix 13). The 16S rRNA gene sequence similarity of strain F130T^T to the remaining *Dietzia* species ranged from 96.26% to 99.70%. This association was supported by all the phylogenetic tree algorithms by a 99% of the bootstrap value in the neighbour-joining analysis (Figure 8.2).

Strain F96T^T appeared to fall within the genus *Leifsonia* clade 99.85% of similarities corresponding to 2 nucleotide differences to *Leifsonia soli* DSM 23871^T (Appendix 13). This association was supported by maximum-likelihood algorithm with a low bootstrap value of 57% in neighbor-joining analysis (Figure 8.3). In comparison, strains F42M^T and F152M^T were found to reside within the genus of *Rhodococcus.* Strain F42M^T possessed 96.88% similarity (42 nucleotide differences) to *Rhodococcus baikonurensis* DSM 44587^T, whilst F152M^T was most closely related to *Rhodococcus jialingiae* DSM 45257^T and *Rhodococcus qingshengii* KCTC 19205^T, with the same percentage similarity value of 98.56%, corresponding to 18 nucleotide differences (Appendix 13). The similarity shared between strains F42M^T and F152M^T was found to be lower at 95.44% with a large nucleotide difference of 57 (Appendix 13). The associations of these strains were also supported by all the tree-making algorithms at a lower bootstrap value of less than 50% in neighbour-joining analysis (Figure 8.4).

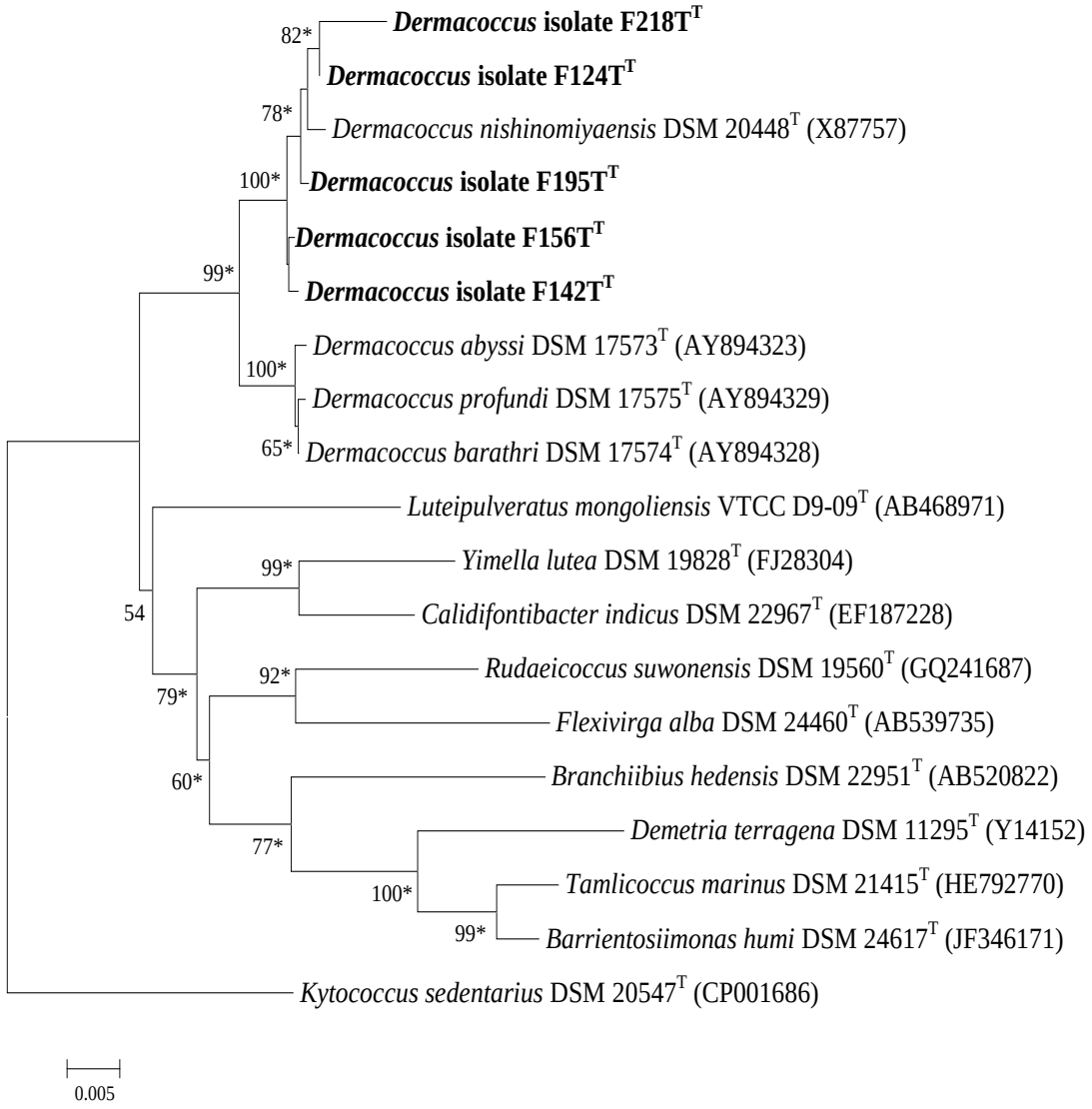


Figure 8.1: Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene sequences of strain F124T^T, F142T^T, F156T^T, F218T^T and F195T^T showing its position in the *Dermacoccus* clade from the representative of the family *Dermacoccaceae*. Asterisks indicate branches of the tree that were found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) algorithms. Bootstraps were calculated with 1000 replications with only values ≥ 50% are shown. The sequence of *Kytococcus sedentarius* DSM 20547^T was used as an out-group. Bar, 0.005 substitutions per nucleotide position.

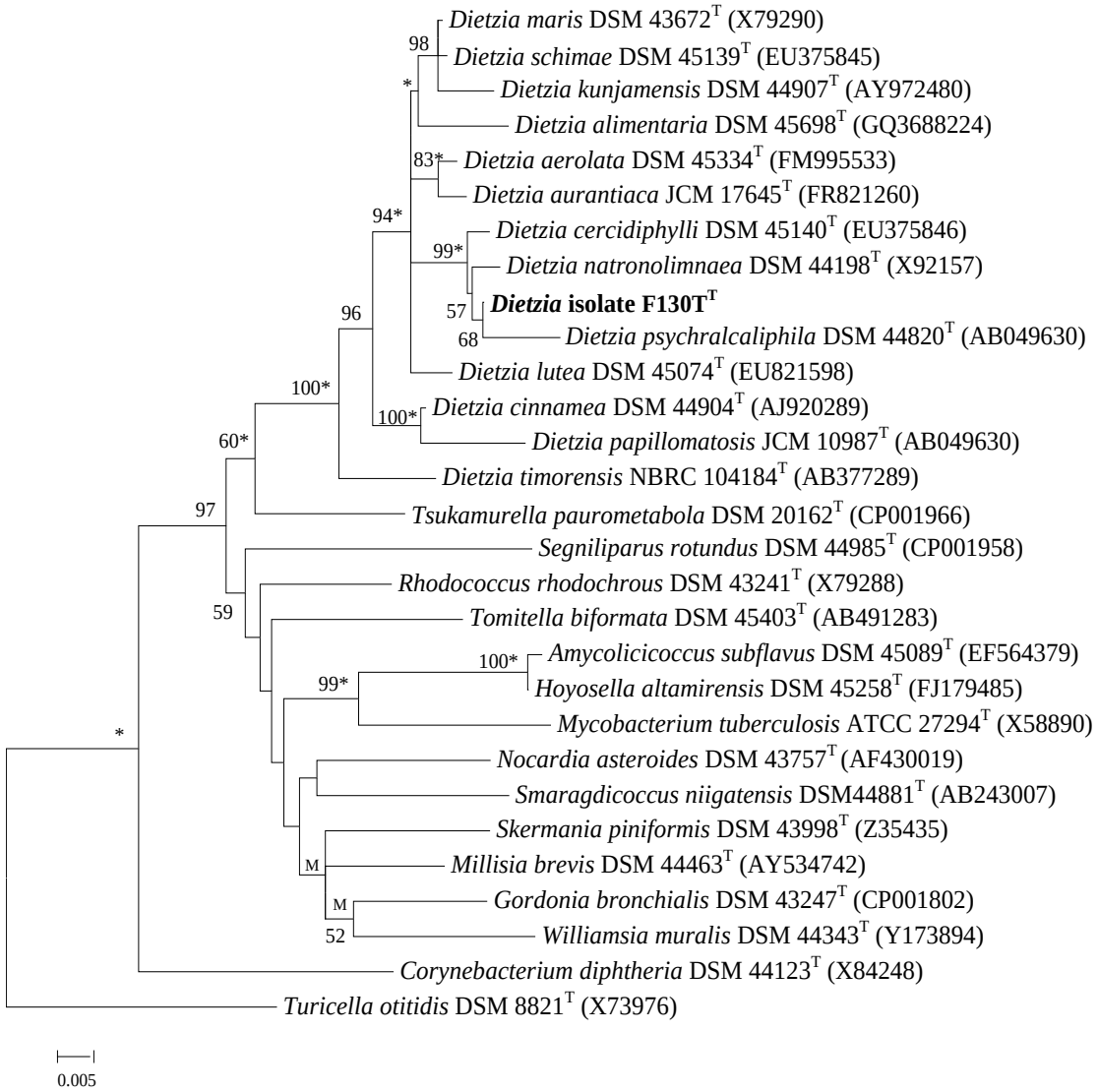


Figure 8.2: Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene sequences of strain F130T^T showing its position in the *Dietzia* clade from the representative of the family *Dietziaceae*. Asterisks indicate branches of the tree that were found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) algorithms. M indicates branches that were found in maximum-likelihood tree. Bootstraps were calculated with 1000 replications with only values ≥ 50% are shown. The sequence of *Turicella otitidis* DSM 8821^T was used as an out-group. Bar, 0.005 substitutions per nucleotide position.

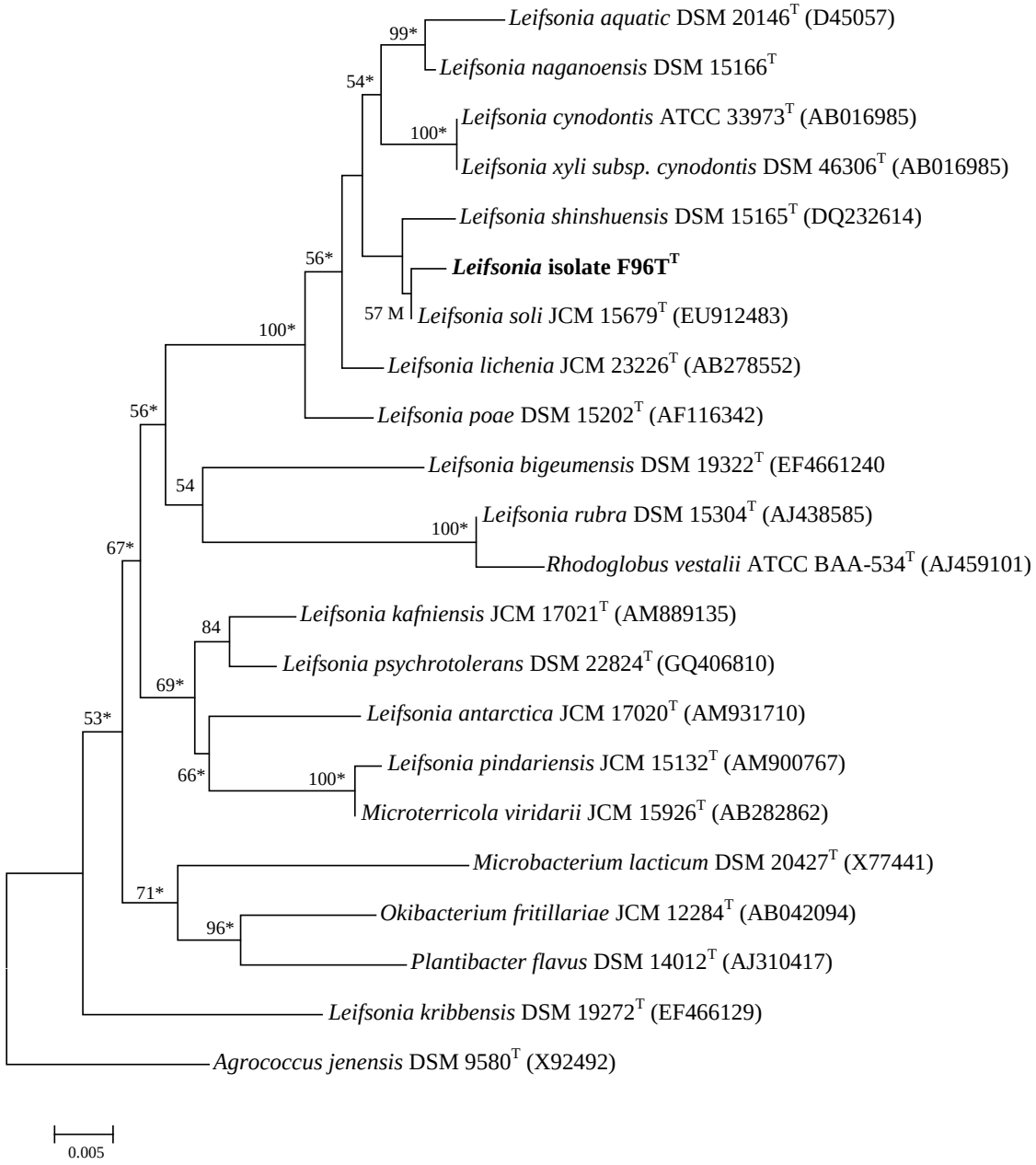


Figure 8.3: Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene sequences of strain F96T^T showing its position in the *Leifsonia* clade from the representative of the family *Microbacteriaceae*. Asterisks indicate branches of the tree that were found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) algorithms. M indicates branches that were found in maximum-likelihood tree. Bootstraps were calculated with 1000 replications with only values ≥ 50% are shown. The sequence of *Agrococcus jenensis* DSM 9580^T was used as an out-group. Bar, 0.005 substitutions per nucleotide position.

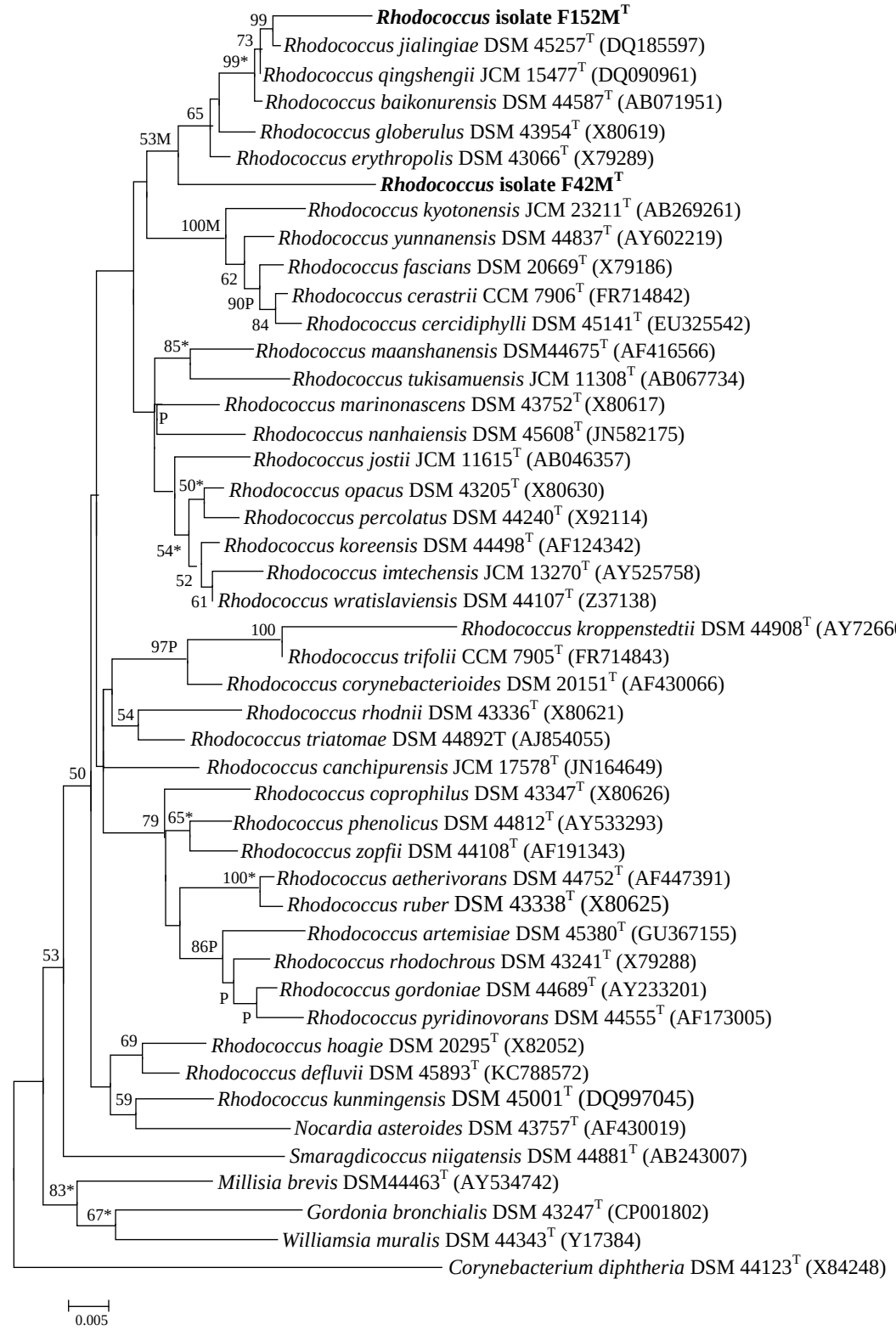


Figure 8.4: Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene sequences of strain F42M^T and F152M^T showing its position in the *Rhodococcus* clade from the representative of the family *Norcadiaceae*. Asterisks indicate branches of the tree that were found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) algorithms. P indicates branches that were found in maximum-parsimony tree. Bootstraps were calculated with 1000 replications with only values ≥ 50% are shown. The sequence of *Corynebacterium diphtheria* DSM 44123^T was used as an out-group. Bar, 0.005 substitutions per nucleotide position.

8.3.2. Chemotaxonomic analysis

Chemotaxonomy analyses were also carried out to ascertain the presence of the mycolic acids methyl esters (MAMES) (Minnikin *et al*., 1975), the identity of fatty acids methyl esters (FAMES) (Sutcliffe, 2000) and polar lipids (Minnikin *et al*., 1984) in all the test strains alongside one marker strain from each genus to serve as a reference. The mycolic acids if present were identified by calculating and comparing to the reported R_f values of the known type strains. Similarly, polar lipids were also determined by comparing to the reported polar lipids patterns of the known neighbouring type strains. Fatty acid methyl esters were identified by comparing to the Supelco 37-Component FAME Mix standard (Sigma-Aldrich; 47885-U) that were analysed together with the test strains.

For *Dermacoccus* related strains of F124T$^{\mathbf{T}}$, F142T$^{\mathbf{T}}$, F156T^{T}, F218T$^{\mathbf{T}}$ and F195T$^{\mathbf{T}}$, mycolic acids were absent on 1D-TLC which was in correspondence to *D. nishinomiyaensis* DSM 43672^{T} (Stackebrandt *et al*., 1995) (Figure 8.5). The polar lipids were identified by comparing to the polar lipids patterns reported by Ruckmani *et al*. (2011) on one of the member from family *Dermacoccaceae*. The major polar lipids detected were phosphatidyl-inositol (PI), diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), alongside several unidentified glycolipids (G) (Figure 8.6a to 8.6f). The FAMES profile of these strains varied slightly (Table 8.1). The fatty acids detected for strains F142T^{T} and F156T^{T} are 14-methylhexadecanoic (ai$C_{17:0}$; 9.1%), monosaturated octadecanoic ($C_{18:1}$; 7.1%), 15-methylhexadecanoic (i$C_{17:0}$; 6.6%), 14-methylpentadecanoic (i$C_{16:0}$; 5.1%), hexadecanoic ($C_{16:0}$; 4.3%), heptadecanoic ($C_{17:0}$; 3.9%), 9-hexadecenoic methyl ester (Z) ($C_{16:1}$; 3.1%), minor proportions of octadecanoic ($C_{18:0}$; 2.3%) and trace amounts of 10-nonadecenoic ($C_{19:1n9}$) and pentadecanoic ($C_{15:0}$) alongside two unknown fatty acids detected at retention time of 37.28 and 38.12. Likewise, these fatty acids were also detected in strain F124T^{T} but with the absence of 14-methylpentadecanoic (i$C_{16:0}$) while for strains F218T^{T} and F195T^{T}, fatty acid 10-nonadecenoic ($C_{19:1n9}$) was not detected.

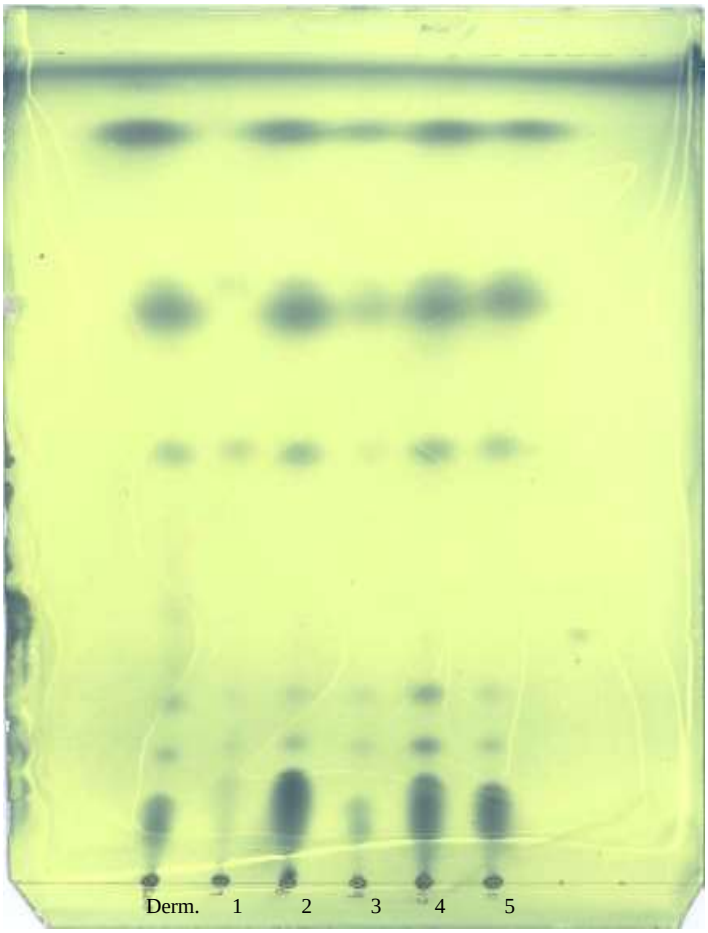


Figure 8.5: Mycolic acids profile of *Dermacoccus* strains which co-migrated with its relative type strains on 1D-TLC. Derm. = *Dermacoccus nishinomiyaensis* DSM 20448^{T}. 1 = F124T^{T}, 2= F142T^{T}, 3= F156T^{T}, 4= F218T^{T} and 5= F195T^{T}

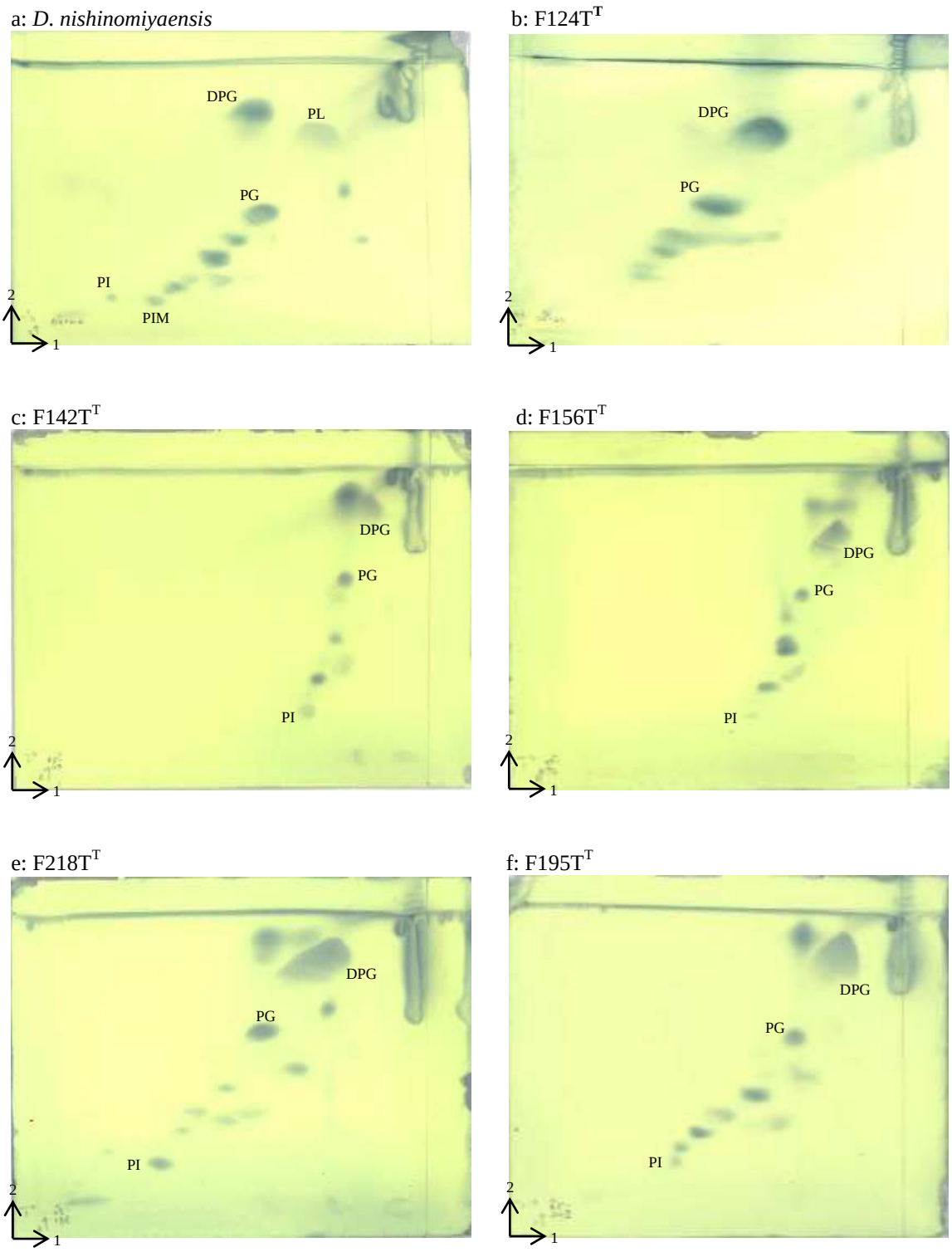


Figure 8.6: Polar lipids profiles analysed using 2D-TLC which were stained with 5% ethanolic molybdophosphoric acid. a) polar lipids profiles of *Dermacoccus nishinomiyaensis* DSM 20448^T, b) F124T^T, c) F142T^T, d) F156T^T, e) F218T^T and f) F195T^T**.** First and second dimensions are indicated at the bottom left-hand corner. Abbreviations: PI, phosphatidyl-inositol, PG, phosphatidylglycerol; DPG, diphosphatidylglycerol, G, glycolipid.

Table 8.1: Percentage fatty acid composition of isolates and the phylogenetically close neighbours

	Fatty acid composition (%)																		
	C14:0	aiC15:0	C15:0	iC16:0	C16:0	C16:1	iC17:0	aiC17:0	Unknown	C17:0	Unknown	iC18:0	C18:0	C18:1	TSA_{18}	C18:2n6c	C19:1n9	C20:0	C20:1n9
RT	31.94	33.40	33.95	34.97	35.82	36.40	36.78	37.05	37.28	37.62	38.12	38.64	39.37	39.80	39.87	40.73	41.45	42.61	43.00
D. nishinomiyaensis DSM 20448^T	trace	trace	trace	3.60	4.30	5.78	11.08	4.62	6.06	5.34	11.60		trace	16.01			trace		
F124T^T	trace		3.46		6.16	trace	10.34	trace	trace	26.93	9.95	4.43	11.82	5.24			2.81	trace	
F142T^T	trace	trace	trace	5.12	4.30	3.06	6.56	9.08	2.63	3.85	6.96	trace	2.25	7.09			trace		
F156T^T	trace	trace	2.73	trace	12.72	trace	3.17	8.43	trace	10.58	3.10	8.64	18.29	3.45			trace		
F218T^T	trace	trace	trace	3.08	3.53	4.18	20.22	4.11	4.87	10.60	20.59		trace	7.88					
F195T^T			trace	5.93	trace	2.37	13.29	3.78	5.87	10.05	11.96	trace	2.66	9.44			trace		
D. maris DSM 43672^T			4.34		22.24	trace				6.19	8.65			16.11					
F130T^T					13.40					70.95						15.65			
L. soli DSM 23871^T		11.79		4.75	trace		trace	76.84											
F96T^T		8.37		7.34	trace		trace	80.47											
R. rhodochrous DSM 43241^T	trace				20.73	2.88				trace			trace		41.86			trace	
F42M^T	3.88		trace		17.85	6.81				trace			trace		39.76		5.03	trace	
F152M^T	4.90		2.43		17.57	trace				trace			trace		38.06			trace	trace

C14:0 = heptadecanoic; aiC15:0 = 12-methyltetradecanoic; C15:0 = pentadecanoic; iC16:0 = 14-methylpentadecanoic; C16:0 = hexadecanoic; C16:1 = 9-hexadecenoic methyl ester (Z); iC17:0 = 15-methylhexadecanoic; aiC17:0 = 14-methylhexadecanoic; C17:0 = heptadecanoic; iC18:0 = 16-methylheptadecanoic; C18:0 = octadecanoic; C18:1 = monosaturated octadecanoic; TSA_{18} = 10-methyloctadecanoic; C18:2n6c = 9, 12(Z, Z)-octadecadienoic; C19:1n9 = 10-nonadecenoic; C20:0 = eicosanoic; C20:1n9 = 11-eicosenoic. Percentage value < 2 is indicated as 'trace'.

For *Dietzia* isolate F130T^T, the mycolic acids identified were in equivalent mobility to those of *D. maris* DSM 43672^T at R_f = 0.26 (Figure 8.7). The position of mycolic acids was also similar to that determined by Yassin *et al*. (2006). By referring to the work done by Kim *et al*. (2011), the polar lipids identified for strain F130T^T were PI, G, PG, DPG, as well as several unknown polar lipids (Figure 8.8a and 8.8b). The fatty acids that were found present were hexadecanoic ($C_{16:0}$; 13.4%), heptadecanoic ($C_{17:0}$; 71.0%) and linoleic acid ($C_{18:2n6c}$; 15.7%) (Table 8.1).

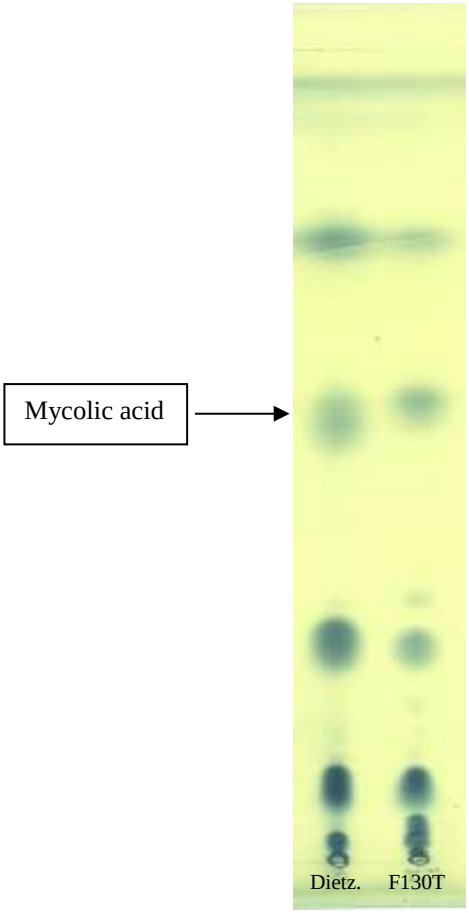


Figure 8.7: Mycolic acids profile of *Dietzia* strain F130T^T which co-migrated with its relative type strain on 1D-TLC. Dietz. = *Dietzia maris* DSM 43672^T. Arrow indicates the position of mycolic acid.

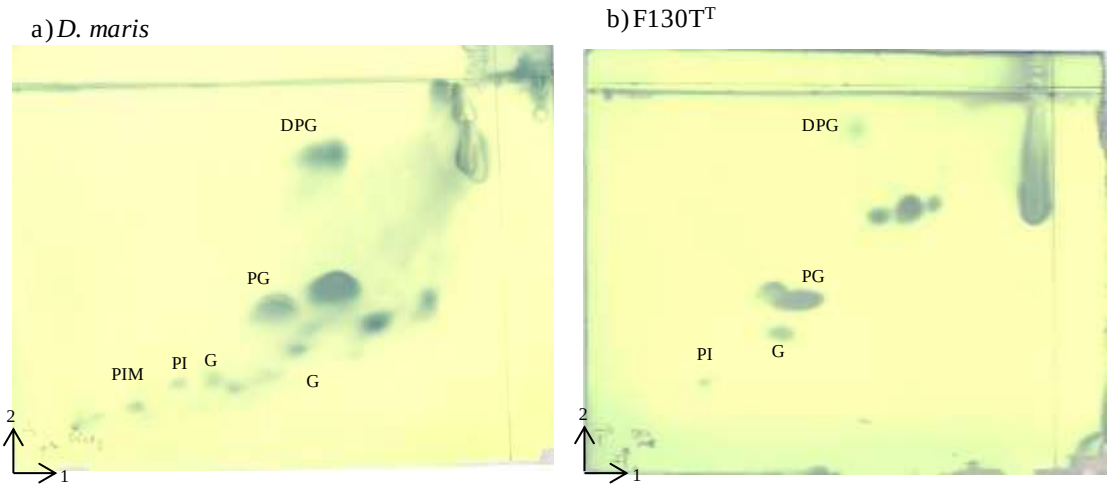
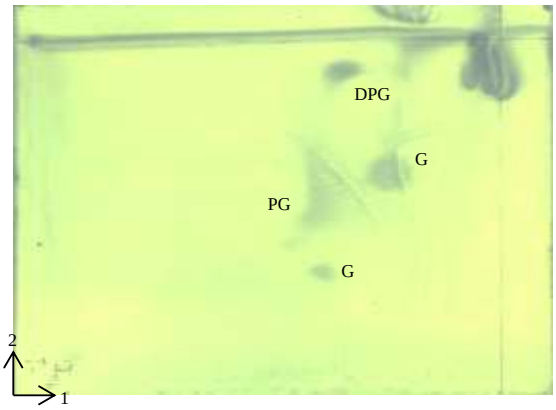


Figure 8.8: Polar lipids profiles of *Dietzia* strains which were analysed using 2D-TLC and stained with 5% ethanolic molybdophosphoric acid. a) *Dietzia maris* DSM 43672^T, b) F130T^T. First and second dimensions are indicated at the bottom left-hand corner. Abbreviations: PIM, phosphatidylinositol mannoside PI, phosphatidyl-inositol, G, glycolipid, PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

Strain F96T^T was found to not contain mycolic acids, which was in agreement to the reference type strain, *L. soli* DSM 23871^T (Madhaiyan *et al*., 2010) (Figure 8.9). Besides, the major polar lipids detected were PG, DPG and G (Figure 8.10a and 8.10b) which is comparable to those reported by Pindi *et al*. (2009). The strains fatty acid methyl ester profile was found to contain major amounts of 14-methylhexadecanoic (aiC$_{17:0}$) acid at 80.5% followed by 12-methyltetradecanoic (aiC$_{15:0}$; 8.4%) and 14-methylpentadecanoic (iC$_{16:0}$; 7.3%) acids (Table 8.1).

Figure 8.9: Mycolic acids profile of *Leifsonia* strain F96T^T on 1D-TLC.
Leif. = *Leifsonia soli* DSM 23871^T.

a) *L. soli*



b) F96T^T

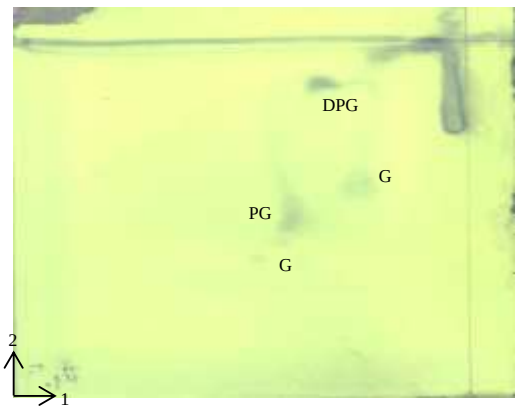


Figure 8.10: Polar lipids profiles of *Leifsonia* strains which were analysed using 2D-TLC and stained with 5% ethanolic molybdophosphoric acid. a) *Leifsonia soli* DSM 23871^T, b) F96T^T. First and second dimensions are indicated at the bottom left-hand corner. Abbreviations: G, glycolipid, PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

For the rhodococcal isolates F42M^T and F152M^T, the migration of mycolic acids was in equivalent mobility to those of *R. rhodochrous* DSM 43241^T at an R_f 0.29 (Figure 8.11). The position of the mycolic acids was comparable to that determined by (Kämpfer *et al.*, 2013). The major polar lipids identified were phosphatidylethanolamine (PE), PIM, PI, PG, DPG, PL and several unknown polar lipids which is in correspondence to those reported by Zhao *et al*. (2012) (Figure 8.12a to 8.12c). Both strains contained the following fatty acid methyl esters; 10-methyloctadecanoic (TSA_{18}; 38.1%), hexadecanoic ($C_{16:0}$; 17.6%), tetradecanoic (C_{14}; 4.9%), pentadecanoic (C_{15}; 2.4%), minor portions of heptadecanoic ($C_{17:0}$), 9Z-hexadecenoic ($C_{16:1}$), octadecanoic ($C_{18:0}$) and eicosanoic ($C_{20:0}$) acids (Table 8.1). Strain F42M^T was found to contain 5.0% of 10-nonadecenoic ($C_{19:1n9}$) compared to strain F152^T which contained minor portions of 11-eicosenoic ($C_{20:1n9}$) (Table 8.1).

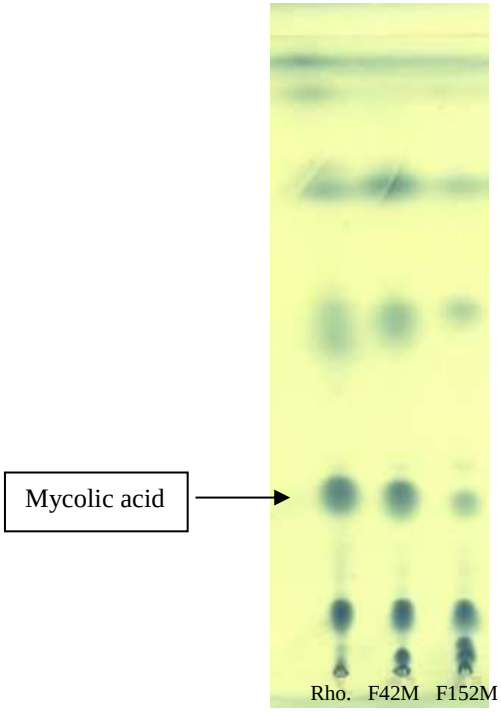
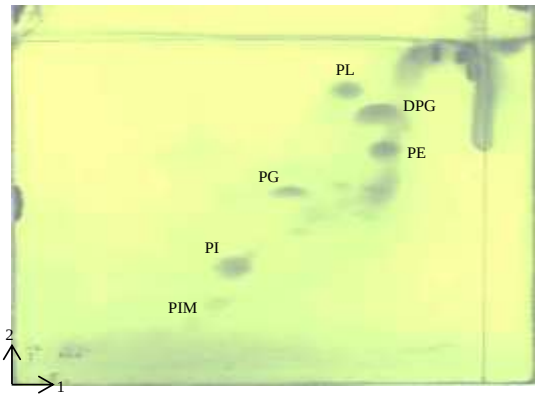
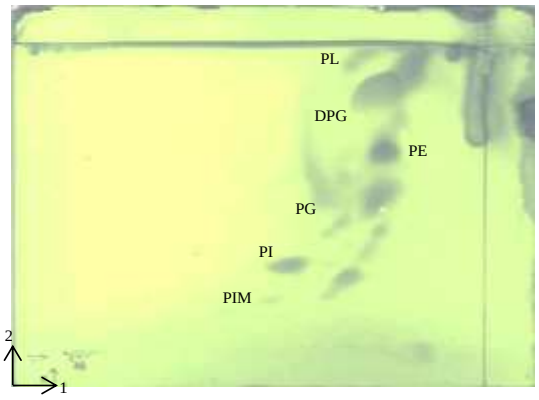


Figure 8.11: Mycolic acids profile of *Rhodococcus* strains F42M^T and F152M^T on 1D-TLC. Rho. = *Rhodococcus rhodochrous* DSM 43241^T. Arrow indicates the position of mycolic acid.

a) *R. rhodochrous*



b) F42M^T



c) F152M^T

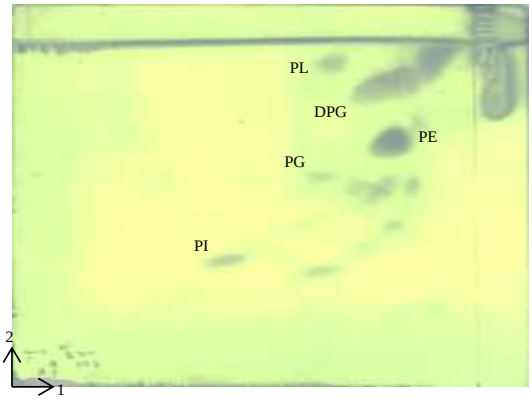


Figure 8.12: Polar lipids profiles of *Rhodococcus* strains which were analysed using 2D-TLC and stained with 5% ethanolic molybdophosphoric acid. a) *Rhodococcus rhodochrous* DSM 43241^T, b) F42M^T, c) F152M^T. First and second dimensions are indicated at the bottom left-hand corner. Abbreviations: PIM, phosphatidylinositol mannoside PI, phosphatidyl-inositol, PG, phosphatidylglycerol; DPG, diphosphatidylglycerol, PL, phospholipid, PE, phosphatidylethanolamine.

8.3.3. Phenotypic characterisation

Biochemical, degradation, enzymatic reactions and nutritional tests were carried out for each of the test strains alongside the neighbouring type strains to further characterise and differentiate between each other (Appendix 10). Table 8.2 illustrates the difference between the isoaltes and their closest neighbours. In Table 8.2, strains F124T, F142T, F156T, F218T, F195T and *D. nishinomiyaensis* DSM 43672^{T} shared quite a number of common properties but in certain phenotypic tests, they were found different. *D. nishinomiyaensis* DSM 43672^{T} compared to the isolates was able to grow at pH 8.5, pH 9, pH 10 but not at 45°C; did not degrade allantoin, arbutin and starch; did not produce acid from D (+) mannose; utilises 4-aminobutyrate, starch (all at 1%; w/v), D-glucuronic acid, pimelic acid (all at 0.1%; w/v) as sole carbon sources for energy and growth but not aesculin, arbutin, guanine, lactose, L-arabitol, *myo*-inositol, methyl caprate (all at 1%; w/v) and sebacic acid (0.1%; w/v); also utilised acetamide, DL-norleucine and ethanolamine as sole carbon and nitrogen sources (0.1%; w/v) but not L-histidine and L-phenylalanine. Table 8.4 demonstrates that these strains could also be differentiated from each other and between *D. abyssi*, *D. barathri* and *D. profundi* through the series of phenotypic analyses.

In addition, phenotypic analysis showed that strain F130T^{T} did it possess properties in line with the species within the genus of *Dietzia*, strain F130T^{T} could also be readily distinguished from its closest related type strain of *D. cercidiphylli* by its ability to degrade aesculin but not elastin; able to utilise butane-1,4-diol; D-galactose, L-sorbose (all at 1.0 %; w/v), D-galacturonic acid, D-mandelic acid, fumaric acid and DL-lactic acid, sebacic acid (all at 0.1%; w/v) but not D-glucosamine HCl and guanine (all at 1.0 %; w/v) as sole carbon sources; utilise L-tryptophan but not L-cysteine, L-histidine and L-serine as sole carbon and nitrogen sources (0.1%; w/v). Other additional phenotypic properties between other *Dietzia* species were also presented in Table 8.3.

Strain F96T^{T} demonstrated a wide range of phenotypic properties which were in line with the type strains within the genus *Leifsonia* as shown in Table 8.4. Particularly, strain F96T^{T} could be distinguished from its closest relative, *L. soli* DSM 23871^{T} by its ability to grow at pH 9 but not at pH 4.5, on 7% NaCl and 7.5% NaCl. Strain F96T^{T} was able to degrade elastin but not uric acid; did not produce acid from α-L-rhamnose; utilises cellulose, glycogen, L-xylose, starch (all at 1%; w/v), sodium acetate, sodium

malonate and spermine (all at 0.1%; w/v) as sole carbon sources but not hypoxanthine, L-alanine (all at 1%; w/v), and sodium benzoate (0.1%; w/v); possessed the ability to utilise DL-methionine, DL-norvaline, glycine and L-proline but not L-alanine as sole carbon and nitrogen sources (0.1%; w/v). Other *Leifsonia* species can also be differentiated from strain F96T through varying phenotypic properties as shown in Table 8.4.

Strain F42M^{T} could also be distinguished from its closest neighbour *Rhodococcus baikonurensis* DSM 44587^{T} (Table 8.5). Strain F42M^{T} differed from *R. baikonurensis* as it was able to grow in 9 and 10% salt concentrations; resistance to lysozyme; its capability to degrade gelatin, L-tyrosine, Tween 40 and 60 and starch, but not elastin and hypoxanthine; did not hydrolyse arbutin or produce acid from D (+) mannose and α-L-rhamnose; utilised 4-aminobutyrate, adonitol, aesculin, casein (all at 1.0%; w/v), D-glucuronic acid, sodium malate, spermine (all at 0.1%; w/v) but not lactose, L-sorbose (all at 1.0%; w/v), D-gluconic acid and paraffin (all at 0.1%; w/v) as sole carbon sources and energy; also utilised DL-methionine, L-cysteine, L-lysine, L-proline, urea but not glycine, L-glutamine and L-tryptophan as sole carbon and nitrogen sources (0.1%; w/v).

Strain F152M^{T} could also be differentiated from *R. jialingiae* DSM 43257^{T} and *R. qingshengii* KCTC 19205^{T} from its ability to degrade L-tyrosine, Tween 40; to produce acid from maltose but not melibiose; utilised L-sorbose, L-xylose, methyl α-D mannopyranoside, methyl β-D-xylopyranoside (all at 1.0%, w/v), D-galacturonic acid, D-glucuronic acid, *p*-hydroxybenzoic acid, sebacic acid, sodium citrate, sodium tartrate, valeric acid and vanillin (all at 0.1%; w/v) but not D-gluconic acid (0.1%; w/v) as sole carbon sources; utilised L-cysteine, L-tryptophan but not L-glutamine as sole carbon and nitrogen sources (0.1%; w/v) (Table 8.5). Strain F42M^{T} and F152M^{T} could also be distinguished from each other and other *Rhodococcus* species via other phenotypic properties as presented in Table 8.5.

Table 8.2 Phenotypic properties that distinguish strain F124T^T, F142T^T, F156T^T, F218T^T and F195T^T from the type strains of *Dermacoccus* species

	F124T^T	F142T^T	F156T^T	F218T^T	F195T^T	*D. abbysi* DSM 17573^T	*D. barathri* DSM 17574^T	*D. nishinomiyaensis* DSM 20448^T	*D. profundi* DSM 17575^T
Growth at:									
45°C	+	+	+	+	+	-	-	-	-
pH 4	+	-	-	-	-	-	-	+	-
pH 4.5	+	+	+	+	+	+	+	+	-
pH 7.5	-	-	+	+	-	+	+	+	+
pH 8.5	-	-	-	-	-	+	+	+	+
pH 9	-	-	-	-	-	+	+	+	+
pH 10	-	-	-	-	-	+	+	+	+
9.0% NaCl	-	-	-	-	-	+	+	+	+
10.0% NaCl	-	-	-	-	-	+	+	+	+
Biochemical tests:									
Hydrogen sulfide	+	-	-	-	-	-	-	-	-
Lysozyme	+	+	-	+	-	+	+	+	+
Degradation tests:									
0.1% Aesculin	+	-	-	+	-	+	+	+	+
1% Allantoin	+	+	+	+	+	+	+	-	+
0.1% Arbutin	+	+	+	+	+	-	-	-	-
0.3% Elastin	-	-	-	-	-	-	+	-	+
0.1% Starch	+	+	+	+	+	-	-	-	-
0.5% Uric acid	+	+	-	+	+	+	+	+	+
0.4% Xanthine	+	+	+	+	+	-	+	+	+
Acid production 0.5%; w/v									
D (+) mannose	+	+	+	+	+	-	-	-	-
Maltose	-	-	-	-	+	+	+	+	+
C source 1.0%; w/v									
1,2 propanediol	+	+	+	+	+	+	-	+	+
4-aminobutyrate	-	-	-	-	-	-	+	+	+
Aesculin	+	+	+	+	+	-	-	-	-
Arbutin	+	+	+	+	+	-	+	-	+
Butane-1, 4-diol	-	+	+	+	+	+	+	+	+
D-cellobiose	+	+	+	+	+	-	-	+	+

	F124T^{T}	**F142T^{T}**	**F156T^{T}**	**F218T^{T}**	**F195T^{T}**	***D. abbysi* DSM 17573^{T}**	***D. barathri* DSM 17574^{T}**	***D. nishinomiyaensis* DSM 20448^{T}**	***D. profundi* DSM 17575^{T}**
D-glucosamine HCl	-	-	+	+	+	-	-	-	-
D (-) salicin	+	+	+	+	-	+	+	+	+
D-xylose	+	+	+	+	+	-	+	+	+
Elastin	+	-	-	-	-	+	+	+	+
Guanine	+	+	+	+	+	+	+	-	-
Hypoxanthine	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	-	+	-	-
L-arabitol	+	+	+	+	+	+	+	-	+
myo-inositol	+	+	+	+	+	-	+	-	+
Methyl caprate	+	+	+	+	+	-	+	-	+
Starch	-	-	-	-	-	+	+	+	+
C source 0.1%; w/v									
D-glucuronic acid	-	-	-	-	-	-	+	+	-
D-gluconic acid	+	+	+	-	+	+	+	+	+
L-malic acid	-	-	-	-	-	+	+	-	+
p-hydroxybenzoic acid	-	-	-	-	-	-	-	-	+
Pimelic acid	-	-	-	-	-	+	-	+	-
Sebacic acid	+	-	-	-	+	-	-	-	-
Sodium benzoate	+	-	-	+	+	+	+	+	+
C & N source 0.1%; w/v									
Acetamide	-	-	-	-	-	+	+	+	+
Benzamide	-	+	+	+	+	-	-	+	-
DL-methionine	-	-	-	-	+	+	+	+	+
DL-norleucine	-	-	-	-	-	+	+	+	+
Ethanolamine	-	-	-	-	-	+	+	+	+
L-arginine	-	+	+	+	+	+	+	+	+
L-asparagine	-	-	+	-	-	+	+	+	+
L-aspartic acid	+	-	-	+	-	-	-	-	-
L-glutamic acid	-	-	-	-	+	-	-	-	-
L-histidine	+	+	+	+	+	+	-	-	-
L-leucine	+	+	+	+	+	+	-	+	+
L-phenylalanine	+	+	+	+	+	+	+	-	+
L-serine	-	+	+	+	+	-	-	+	-

	F124T^T	**F142T^T**	**F156T^T**	**F218T^T**	**F195T^T**	***D. abbysi* DSM 17573^T**	***D. barathri* DSM 17574^T**	***D. nishinomiyaensis* DSM 20448^T**	***D. profundi* DSM 17575^T**
L-tyrosine	+	+	+	+	-	+	+	+	+
Urea	-	-	-	-	-	+	-	-	+
API enzymatic reactions:									
Alkaline phosphatase	+	+	-	+	+	+	-	-	+
Esterase (C4)	+	+	-	+	-	-	-	-	+
Lipase (C14)	-	-	+	-	+	-	-	-	+
Valine arylamidase	-	-	-	+	-	-	-	-	-
Cystine arylamidase	+	+	+	+	-	+	-	+	-
Trypsin	-	-	-	-	+	-	-	-	-
Acid phosphatase	+	+	+	+	+	+	+	+	-
Naphthol-AS-BI-phosphohydrolysase	-	-	-	+	-	-	-	-	-
α-glucosidase	+	+	+	+	+	-	-	+	-

Data for all the strains were obtained from this study. +, positive; -, negative

Table 8.3 Phenotypic properties that distinguish strain F130T^T from the type strains of *Dietzia* species

	F130T^T	*D. cercidiphylli* DSM 45140^T	*D. aerolata* DSM 45334^T	*D. alimentaria* DSM 45698^T	*D. cinnamea* DSM 44904^T	*D. dagingensis* (unpublished)	*D. kunjanmensis* DSM 44907^T	*D. lutea* DSM 45074^T	*D. maris* DSM 43672^T	*D. natronolimnaea* DSM 44198^T	*D. papillomatosis* JCM 10987^T	*D. psychralcaliphilla* DSM 44820^T	*D. shimae* DSM 45139^T	*D. timorensis* NBRC 104184^T
Biochemical test:														
Nitrite reduction	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Degradation tests:														
0.4% Adenine	-	-	-	-	-	+	+	-	-	-	-	-	-	-
0.1% Aesculin	+	-	+	-	-	+	+	+	+	+	+	+	+	+
0.1% Arbutin	+	+	+	+	+	+	-	+	+	+	+	-	-	-
1.0% Cellulose	-	-	-	-	-	+	+	-	-	-	-	-	-	-
0.3% DNA	-	-	-	-	-	-	-	-	-	-	+	-	+	-
0.3% Elastin	+	-	-	-	-	-	-	-	+	+	+	+	+	+
0.4% Xanthine	-	-	-	-	-	+	+	+	+	-	+	+	+	+
Acid production 0.5%; w/v														
D (-) salicin	-	-	-	-	-	+	+	+	+	+	+	+	+	+
D (+) mannose	-	-	-	-	-	+	+	+	+	+	+	+	+	+
D (+) xylose	-	-	+	+	+	+	+	+	+	+	+	+	+	+
C source 1.0%; w/v														
1, 2 propanediol	+	+	+	+	+	-	-	-	-	-	-	-	-	-
4-aminobutyrate	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Butane-1, 4-diol	+	-	+	-	+	+	+	+	+	+	+	+	+	-
Cellulose	+	+	+	+	+	+	+	+	+	-	+	+	+	+
D-cellobiose	+	+	+	+	+	-	-	+	-	+	+	+	+	+
D-galactose	+	-	+	+	+	+	+	+	+	+	+	+	+	+
D- glucosamine HCl	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D-melezitose	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-raffinose	+	+	+	-	+	+	+	+	+	+	+	+	+	+
D (-) salicin	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Elastin	+	+	+	-	-	+	+	+	+	+	+	+	+	+

	F130T^{T}	***D. cercidiphylli* DSM 45140^{T}**	***D. aerolata* DSM 45334^{T}**	***D. alimentaria* DSM 45698^{T}**	***D. cinnamea* DSM 44904^{T}**	***D. dagingensis* (unpublished)**	***D. kunjanmensis* DSM 44907^{T}**	***D. lutea* DSM 45074^{T}**	***D. maris* DSM 43672^{T}**	***D. natronolimnaea* DSM 44198^{T}**	***D. papillomatosis* JCM 10987^{T}**	***D. psychralcaliphilla* DSM 44820^{T}**	***D. shimae* DSM 45139^{T}**	***D. timorensis* NBRC 104184^{T}**
Glycerol	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Hypoxanthine	-	-	-	-	-	+	+	+	-	+	-	-	-	-
Lactose	+	+	+	+	-	-	-	+	+	+	+	+	+	+
L-arabinose	+	+	+	+	+	+	+	+	+	-	+	+	+	+
L-sorbose	+	-	-	-	-	-	-	-	-	-	-	-	-	-
L-xylose	+	+	+	+	+	-	-	-	-	-	-	-	-	-
myo-inositiol	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	-	+	-	+	+	+	+	+
C source 0.1%; w/v														
Adipic acid	+	+	+	+	-	-	-	-	-	-	-	-	-	-
D-galacturonic acid	+	-	+	+	+	-	-	-	-	-	-	-	-	-
D-glucuronic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-mandelic acid	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Fumaric acid	+	-	-	-	-	-	-	-	-	-	-	-	-	-
D-gluconic acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Glutaric acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
DL-lactic acid	+	-	-	+	-	-	-	-	-	-	-	-	-	-
L-malic acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
p-hydroxybenzoic acid	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Pimelic acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Sebacic acid	+	-	+	+	-	-	-	-	+	-	+	+	-	-
Sodium formate	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sodium glutamate	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Sodium malate	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Sodium malonate	-	-	-	-	-	+	-	+	-	+	+	+	+	+
Sodium propionate	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Spermine	-	-	+	-	-	+	+	+	+	+	+	+	+	+

	F130T^{T}	***D. cercidiphylli* DSM 45140^{T}**	***D. aerolata* DSM 45334^{T}**	***D. alimentaria* DSM 45698^{T}**	***D. cinnamea* DSM 44904^{T}**	***D. dagingensis* (unpublished)**	***D. kunjanmensis* DSM 44907^{T}**	***D. lutea* DSM 45074^{T}**	***D. maris* DSM 43672^{T}**	***D. natronolimnaea* DSM 44198^{T}**	***D. papillomatosis* JCM 10987^{T}**	***D. psychralcaliphilla* DSM 44820^{T}**	***D. shimae* DSM 45139^{T}**	***D. timorensis* NBRC 104184^{T}**
Vanillin	-	-	-	-	-	+	-	+	+	-	+	+	-	-
C & N source 0.1%; w/v														
Benzamide	+	+	+	+	-	+	+	+	+	+	+	+	+	+
DL-methionine	+	+	+	-	+	-	+	+	-	-	-	-	+	-
DL-norvaline	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Ethanolamine	+	+	+	+	+	-	-	-	-	-	-	-	-	-
L-asparagine	+	+	+	+	+	+	+	+	+	+	-	+	+	+
L-aspartic acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
L-cysteine	-	+	-	-	-	-	-	-	-	-	-	-	-	-
L-glutamic acid	+	+	+	+	+	-	-	-	-	-	-	-	-	-
L-histidine	-	+	+	+	+	+	+	+	+	+	+	+	+	+
L-serine	-	+	-	-	+	+	+	+	+	+	-	+	+	+
L-tryptophan	+	-	+	+	+	+	+	+	+	+	+	+	+	+
L-tyrosine	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Urea	+	+	-	+	-	+	+	+	+	+	+	+	+	+
API enzymatic reactions:														
Esterase (C4)	+	+	-	-	+	-	-	-	-	-	-	-	+	-
Esterase Lipase (C8)	+	+	+	-	+	+	+	+	-	+	-	-	-	-
Lipase (C14)	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Leucine arylamidase	+	-	+	-	+	+	+	+	+	-	+	-	+	+
Valine arylamidase	+	-	-	-	-	-	-	+	-	-	-	-	-	-
Cystine arylamidase	+	+	-	-	-	+	-	+	-	-	-	+	+	+
Trypsin	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Acid phosphatase	+	+	+	-	+	-	-	+	-	-	+	+	-	+
Naphthol-AS-BI-phosphohydrolysase	-	-	+	-	-	-	-	-	-	-	-	-	-	-
α-glucosidase	-	+	-	-	-	+	+	+	+	+	-	+	+	+
β-glucosidase	+	-	+	-	-	-	-	-	-	-	-	-	-	-

Data for all the strains were obtained from this study. +, positive; -, negative

Table 8.4 Phenotypic properties that distinguish strain F96T^T from the type strains of *Leifsonia* species

	F96T^T	*L. soli* JCM 15679^T	*L. nagano* DSM 15166^T	*L. poae* DSM 15202^T	*L. shinshuensis* DSM 15165^T
Growth at:					
pH 4	-	-	+	+	+
pH 4.5	-	+	+	+	+
pH 8.5	-	-	+	-	-
pH 9	+	-	+	-	-
pH 10	-	-	+	-	-
6.0% NaCl	+	+	+	-	-
7.0% NaCl	-	+	+	-	-
7.5% NaCl	-	+	-	-	-
Lysozyme	+	+	+	+	-
Methyl red	+	+	-	+	-
Degradation tests:					
0.1% Arbutin	-	-	+	+	+
0.3% DNA	+	+	+	+	-
0.3% Elastin	-	+	-	-	-
0.3% RNA	+	+	-	+	-
0.1% Starch	-	-	+	+	+
1.0% Tween 40	+	+	-	-	-
0.5% Uric acid	+	-	-	-	-
Acid production 0.5%; w/v					
α-L-rhamnose	+	-	-	-	-
C source 1.0%; w/v					
1, 2- propanediol	-	-	+	+	+
Adonitol	+	+	+	-	+
Aesculin	+	+	+	+	-
Butane-1, 4-diol	-	-	+	+	+
Cellulose	-	+	-	-	-
D-galactose	+	+	+	-	+
D-mannitol	+	+	+	+	-
D-melibiose	+	+	+	+	-
D-raffinose	+	+	+	+	-

	F96T^T	***L. soli* JCM 15679^T**	***L. nagano* DSM 15166^T**	***L. poae* DSM 15202^T**	***L. shinshuensis* DSM 15165^T**
Elastin	+	+	+	+	-
Glycogen	-	+	+	+	-
Hypoxanthine	+	-	-	-	-
Inulin	+	+	+	-	+
Lactose	+	+	+	+	-
L-alanine	+	-	+	-	+
L-sorbose	-	-	+	-	-
L-xylose	-	+	+	+	+
myo-inositol	-	-	+	+	+
Starch	-	+	+	+	-
C source 0.1%; w/v					
Sodium acetate	-	+	+	+	+
Sodium benzoate	+	-	+	-	-
Sodium malonate	-	+	-	+	-
Spermine	-	+	+	-	-
C & N 0.1%; w/v					
DL-methionine	-	+	-	-	-
DL-norleucine	-	-	+	-	-
DL-norvaline	-	+	+	-	-
Glycine	-	+	-	-	-
L-alanine	+	-	-	-	+
L-cysteine	-	-	-	-	-
L-glutamine	-	-	-	+	-
L-isoleucine	-	-	-	+	-
L-ornithine	-	-	+	+	+
L-proline	-	+	-	+	+
L-theonine	-	-	-	+	-
L-valine	-	-	+	-	-
Urea	-	-	-	-	-
API enzymatic reactions:					
Alkaline phosphatase	-	+	+	+	+
Esterase (C4)	-	-	+	+	+

	F96T^{T}	***L. soli* JCM 15679^{T}**	***L. nagano* DSM 15166^{T}**	***L. poae* DSM 15202^{T}**	***L. shinshuensis* DSM 15165^{T}**
Esterase Lipase (C8)	-	-	-	-	-
Valine arylamidase	+	+	-	+	+
Trypsin	-	-	+	-	+
α-galactosidase	-	+	-	-	+
α-mannosidase	-	+	-	-	+
α-fucosidase	+	+	-	-	-

Data for all the strains were obtained from this study. +, positive; -, negative

Table 8.5 Phenotypic properties that distinguish strain F42M^T and F152M^T from the type strains of *Rhodococcus* species

	F42M^T	*R. baikonurensis* DSM 44587^T	F152M^T	*R. jialingiae* DSM 45257^T	*R. qingshengii* JCM 15477^T	*R. cercidiphylli* DSM 45141^T	*R. erythropolis* DSM 43066^T	*R. jostii* JCM 11615^T	*R. artemisiae* DSM 45380^T	*R. globerulus* DSM 43954^T	*R. nanhaiensis* DSM 45608^T
Growth at:											
37°C	+	+	+	+	+	-	+	+	+	+	+
pH 9	+	+	+	+	+	+	+	+	+	+	-
pH 10	+	+	+	+	+	+	+	+	+	+	-
7.0% NaCl	+	+	+	+	+	+	+	+	+	-	+
7.5% NaCl	+	+	+	+	+	+	+	+	+	-	+
8.0% NaCl	+	+	-	+	-	+	+	-	+	-	-
9.0% NaCl	+	-	-	-	-	+	+	-	+	-	-
10.0% NaCl	+	-	-	-	-	-	-	-	+	-	-
Biochemical tests:											
Hydrogen sulphide	+	+	+	+	+	+	+	+	+	+	-
Lysozyme	+	-	+	+	+	+	+	+	+	-	-
Degradation tests:											
0.1% Aesculin	+	+	+	+	+	+	+	+	-	-	-
1.0% Allantoin	+	+	+	+	+	+	+	+	-	+	-
0.1% Arbutin	-	+	+	+	+	+	+	-	-	+	-
1.0% Cellulose	-	-	-	-	+	-	-	+	-	-	-
0.3% DNA	-	-	-	-	-	-	-	-	-	+	-
0.4% Gelatin	+	-	-	-	-	-	-	-	+	-	-
0.4% Hypoxanthine	-	+	+	+	+	+	+	+	-	+	-
0.5% L-tyrosine	+	-	+	-	-	-	-	-	-	-	-
0.3% RNA	+	+	+	+	-	-	+	-	-	+	-
0.1% Starch	+	-	-	-	-	-	-	-	-	-	+
1.0% Tributyrin	-	-	-	-	-	-	-	-	+	-	-
1.0% Tween 20	-	-	+	-	+	-	+	+	-	+	+
1.0% Tween 40	+	-	+	-	-	-	+	-	+	+	+

	F42M^T	***R. baikonurensis* DSM 44587^T**	**F152M^T**	***R. jialingiae* DSM 45257^T**	***R. qingshengii* JCM 15477^T**	***R. cercidiphylli* DSM 45141^T**	***R. erythropolis* DSM 43066^T**	***R. jostii* JCM 11615^T**	***R. artemisiae* DSM 45380^T**	***R. globerulus* DSM 43954^T**	***R. nanhaiensis* DSM 45608^T**
1.0% Tween 60	+	-	+	-	+	-	+	-	-	+	+
Acid production 0.5%; w/v											
0.5% Uric acid	+	+	+	+	-	+	+	+	-	+	+
0.4% Xanthine	+	-	+	+	-	-	-	+	-	-	-
D (-) salicin	+	+	+	+	+	+	+	+	-	-	-
D (+) mannose	-	+	+	+	-	+	-	+	+	+	+
Melibiose	+	+	-	+	+	+	+	+	+	+	+
Maltose	-	-	+	-	-	-	-	-	-	-	-
α-L-rhamnose	-	+	+	+	+	+	+	+	-	-	-
C source 1.0%; w/v											
1,2-propanediol	-	+	+	-	+	+	+	+	+	+	-
4-aminobutyrate	+	-	-	-	-	-	+	-	-	-	-
Adonitol	+	-	+	+	+	-	+	+	+	+	+
Aesculin	+	-	-	-	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+	+	-	+	+
Butane-1,3-diol	+	+	+	+	+	+	+	+	+	-	+
Butane-1,4-diol	+	+	+	-	+	+	+	+	+	+	+
Casein	+	-	+	+	+	+	+	+	+	+	-
Cellulose	+	+	+	-	+	-	+	-	-	-	-
D-alanine	+	+	+	+	+	+	+	-	+	+	+
D-cellobiose	+	+	+	+	+	-	+	+	+	-	-
Dextrin	+	+	+	+	+	+	+	+	+	+	-
D-glucosamine HCl	+	+	+	+	+	+	+	+	+	-	-
D-mannitol	+	+	+	+	+	+	+	+	+	+	-
D-melibiose	+	+	+	+	+	+	+	+	+	-	-
D-raffinose	+	+	+	+	+	+	+	+	+	+	-
D (-) salicin	+	+	+	+	+	+	+	+	+	+	-

	F42M^T	***R. baikonurensis* DSM 44587^T**	**F152M^T**	***R. jialingiae* DSM 45257^T**	***R. qingshengii* JCM 15477^T**	***R. cercidiphylli* DSM 45141^T**	***R. erythropolis* DSM 43066^T**	***R. jostii* JCM 11615^T**	***R. artemisiae* DSM 45380^T**	***R. globerulus* DSM 43954^T**	***R. nanhaiensis* DSM 45608^T**
Dulcitol	+	+	+	+	-	-	+	+	+	+	-
Elastin	+	+	+	+	+	-	+	+	-	+	-
Glycogen	+	+	+	+	+	+	-	+	+	-	-
Guanine	-	-	-	-	-	-	-	-	-	-	+
Hypoxanthine	-	-	+	-	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	+	+	-	-	-
L-arabitol	+	+	+	+	-	+	+	+	+	+	-
L-sorbose	-	+	+	-	-	-	-	-	+	+	-
L-xylose	-	-	+	-	-	+	+	-	+	-	+
Methyl caprate	+	+	+	+	-	+	+	+	+	+	+
Methyl α-D-glucopyranoside	-	+	+	+	+	+	+	+	-	-	-
Methyl α-D-mannopyranoside	-	-	+	-	-	-	-	-	+	+	+
Methyl β-D-xylopyranoside	-	-	+	-	-	-	-	-	+	+	-
N-acetyl D-glucosamine	-	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	-	+	+	-	+	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	-	+
Xanthine	+	+	+	+	+	+	+	+	-	-	-
C source 0.1%; w/v											
Betaine	-	-	+	-	+	-	-	+	-	-	-
D-galacturonic acid	-	-	+	-	-	-	-	-	-	-	-
D-glucuronic acid	+	-	+	-	-	-	+	+	-	-	-
D-gluconic acid	-	+	-	+	+	+	+	+	+	+	+
Paraffin	-	+	+	+	+	+	+	+	+	-	-
p-hydroxybenzoic acid	-	-	+	-	-	-	-	-	-	-	-
Sebacic acid	-	-	+	-	-	-	-	-	+	-	+
Sodium acetate	-	-	+	-	+	-	-	+	+	+	+

	F42M^T	***R. baikonurensis* DSM 44587^T**	**F152M^T**	***R. jialingiae* DSM 45257^T**	***R. qingshengii* JCM 15477^T**	***R. cercidiphylli* DSM 45141^T**	***R. erythropolis* DSM 43066^T**	***R. jostii* JCM 11615^T**	***R. artemisiae* DSM 45380^T**	***R. globerulus* DSM 43954^T**	***R. nanhaiensis* DSM 45608^T**
Sodium benzoate	+	+	+	+	+	+	-	+	+	+	+
Sodium citrate	-	-	+	-	-	-	-	-	-	-	-
Sodium malate	+	-	-	-	-	-	-	-	-	-	-
Sodium tartrate	-	-	+	-	-	-	-	-	-	-	-
Spermine	+	-	-	-	-	-	-	-	+	-	-
Valeric acid	-	-	+	-	-	-	-	-	-	-	-
Vanillin	-	-	+	-	-	-	-	+	+	-	-
C & N source 0.1%; w/v											
Acetamide	+	+	+	+	+	+	+	+	-	-	-
Benzamide	+	+	+	+	+	+	+	+	-	-	-
D-alanine	+	+	+	+	+	+	+	-	+	+	+
D-glucosamine HCl	+	+	+	+	+	+	+	+	+	-	+
DL-methionine	+	-	+	-	+	-	+	+	+	+	+
DL-norleucine	+	+	+	+	+	+	+	+	+	-	-
DL-norvaline	+	+	+	+	+	+	+	-	+	-	+
Ethanolamine	-	-	-	-	-	-	-	-	+	-	-
Glycine	-	+	+	+	+	+	+	+	+	+	-
L-asparagine	+	+	+	+	-	+	+	+	+	+	+
L-asparctic acid	+	+	-	+	+	-	-	+	-	-	-
L-cysteine	+	-	+	-	-	-	-	+	+	-	-
L-glutamic acid	+	+	+	+	+	+	+	+	-	-	-
L-glutamine	-	+	-	+	+	+	+	+	-	-	-
L-histidine	+	+	+	+	+	+	-	-	+	+	+
L-isoleucine	-	-	-	-	-	-	-	-	+	+	-
L-lysine	+	-	+	-	+	+	+	+	+	+	+
L-ornithine	-	-	-	-	-	-	-	-	+	-	-
L-proline	+	-	+	-	+	+	+	+	+	+	+

	F42M^T	***R. baikonurensis* DSM 44587^T**	**F152M^T**	***R. jialingiae* DSM 45257^T**	***R. qingshengii* JCM 15477^T**	***R. cercidiphylli* DSM 45141^T**	***R. erythropolis* DSM 43066^T**	***R. jostii* JCM 11615^T**	***R. artemisiae* DSM 45380^T**	***R. globerulus* DSM 43954^T**	***R. nanhaiensis* DSM 45608^T**
L-threonine	+	+	+	+	+	+	+	+	+	-	-
L-tryptophan	+	-	+	-	-	+	+	+	+	+	+
L-valine	-	-	-	-	-	-	-	-	+	-	+
Potassium nitrate	-	-	+	+	-	+	-	+	-	-	-
Urea	+	-	-	-	-	-	-	-	+	+	-
API ZYM											
Esterase (C4)	+	+	+	-	-	-	+	-	+	+	+
Esterase Lipase (C8)	+	+	+	+	+	+	-	+	+	+	+
Lipase (C14)	-	-	-	-	+	+	-	-	-	+	-
Valine arylamidase	+	+	+	+	+	+	+	+	-	-	+
Cystine arylamidase	+	+	+	+	+	+	+	-	-	+	-
α-chymotrypsin	+	+	+	+	+	+	+	-	-	-	-
Naphthol-AS-BI-phosphohydrolysase	+	+	+	+	+	-	+	-	-	-	+
α-galactosidase	-	-	-	-	-	+	-	-	-	-	-
β-galactosidase	-	-	-	+	+	+	+	+	-	-	-
α-glucosidase	+	+	+	+	+	+	+	+	-	-	+
β-glucosidase	+	+	+	+	+	-	+	+	+	+	-

Data for all the strains were obtained from this study. +, positive; -, negative

8.4. Discussion:

From the phenotypic and phylogenetic analyses, all the test strains were able to be distinguished from their closest type strains. The *Dermacoccus* test strains not only shared a wide range of properties within its respective genus, they could also be distinguished from the type strains by a combination of properties. The phylogenetic consensus tree illustrated that strains F124T^T, F142T^T, F156T^T, F218T^T and F195T^T could be distinguished from the type strains within the genus of *Dermacoccus*.

However, the similarities between the *Dermacoccus* isolates and their related type strains were very high (> 98%) and they were greater than the thresholds set for 16S rRNA sequences similarities as indicated by Stackebrandt and Ebers (2006) at 97% and a more recent report by Kim *et al*. (2014) at 98.65%. The threshold at 98.65% is a value set as an indicative boundary of strains belonging to a different genomic species (Kim *et al*., 2014; Wayne *et al*., 1987). Strains F124T^T, F142T^T, F156T^T and F195T^T were highly similar to the type strains *D. abyssi*, *D. barathri*, *D. nishinomiyaensis* and *D. profundi* while strain F218T^T has shown to be 99.09% similar to *D. nishinomiyaensis* (Appendix 14). In fact, the similarities between these test strains and their neighbouring type strains were greater than the threshold value, thus recognition as novel members of *Dermacoccus* could not be made without whole-genome analysis being carried out (Appendix 14).

Similarly, the similarities of strain F130T^T to its neighbouring type strains of *D. cercidiphylli* (99.7%), *D. natronolimnaea* (99.63%) and *D. psychralcaliphila* (98.81%) were also above the threshold. Likewise, for strain F96T^T which was highly similar to its neighbouring type strains of *L. lichenia* (99.32%), *L. shinshuensis* (99.32%) and *L. soli* (99.85%). Evidently, an additional analysis such as DNA-DNA hybridisation (DDH) or whole-genome sequencing is essential in order to resolve the high similarities between the strains.

DDH is a widely used genotypic analysis that indirectly measures the overall similarity between two genome sequences (McCarthy and Bolton, 1963; Schildkraut *et al*., 1961). This microbial species delineation technique has been the ‘gold standard’ for bacterial species discrimination with species boundary cut-off at 70% (Tindall *et al*., 2010; Wayne *et al*., 1987), corresponding to the 97% threshold of 16S rRNA gene

sequence similarity (Stackebrandt and Goebel, 1994). However, this technique is time-consuming, labour-intensive (Gevers *et al*., 2005; Stackebrandt *et al*., 2002) and often yields different results particularly for lower reassociation values (Auch *et al*., 2010; Kim *et al*., 2014).

Whole-genome sequencing such as the average nucleotide identity (ANI) is an alternative solution to DDH. ANI is the average nucleotide identity of the total genomic sequence shared between two strains (Konstantinidis *et al*., 2006). It is also a robust method for measuring evolutionary relatedness between closely related bacterial strains. Besides, studies have demonstrated that ANI has correlation with DDH, where genomes with ANI value of ≥ 95% is corresponded to the current DDH standard at 70% (Chan *et al*., 2012; Goris *et al*., 2007; Konstantinidis and Tiedje, 2005). This has also indicated that ANI provides a better correlation with 16S rRNA gene sequence similarity in comparison to DDH (Kim *et al*., 2014).

In contrast to the other test strains, isolates F42M^T and F152M^T that belong to the genus of *Rhodococcus* have similarity values below 96.88% and 98.65%, respectively when they were compared between the neighbouring type strains. These values indicated that they deserve the recognition as new species. Besides, they can also be readily distinguished from its members in the genus of *Rhodococcus* based on the phenotypic properties. However, for publication purposes, strain F152M^T has to be re-sequenced due to short 16S rRNA gene sequence length of < 1400 bp (Appendix 5). Therefore, *Rhodococcus* strain F42M^T with 1547 bp 16S rRNA gene sequence length deserves a novel species status and the name proposed for this strain is *Rhodococcus meromictica* sp. nov.

Description of *Rhodococcus meromictica* sp. nov.

Rhodococcus meromictica (meromictica refers to the lake condition where the type strain was being isolated)

Produced circular, raised, undulate and moderate orange pigmented colonies in 0.2 cm diameter. Grew at 10 to 28°C, from pH 4.5 to 9.5. Revealed chemical markers characteristic of the genus Rhodococcus. Catalase positive but oxidase negative. Grew on 2 to 6% salt concentration but not at 15%. Reduced nitrate to nitrite but was negative for indole production, methyl red and Voges–Proskauer tests. Degraded guanine but not

adenine, casein, and xylan. Produced acid from D (-) arabinose, D (+) xylose but not acetamide, adonitol, D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannitol, D-raffinose, D-ribose, D-sorbitol, dulcitol, glycerol, inulin, lactose, L-arabinose, meso-erythritol, myo-inositol, starch, sucrose and trehalose. Utilised 2,3-propanediol, amygdalin, butane-1-ol, D-arabinose, D-arabitol, D-fructose, D-galactose, D-glucose, D-melezitose, D-ribose, D-sorbitol, ethanol, glycerol, inulin, iso-amyl-alcohol, L-alanine, L-arabinose, maltose, meso-erythritol, myo-inositol, Tween 40 (all at 1.0%; w/v or v/v), sodium glutamate, sodium malonate (all at 0.1%; w/v) but not tyramine (1.0%; w/v), acetic acid, adipic acid, anthranilic acid, D-mandelic acid, fumaric acid, glutaric acid, isobutyric acid, DL-lactic acid, L-malic acid, m-hydroxybenzoic acid, pimelic acid, sodium formate, sodium oxalate, sodium propionate, sodium pyruvate and sodium succinate (all at 0.1%; w/v) as sole carbon sources and energy. Utilised gelatin, L-alanine, L-arginine, L-leucine, L-phenylalanine, L-serine and L-tyrosine as sole carbon and nitrogen sources (0.1%; w/v). Additional phenotypic properties are shown in Table 8.5.

The type strain F152M^{T} (DSM…….^{T}; NCIMB…….^{T}), was isolated from the freshwater sediment of Lake Suigetsu, Japan, at depth 1363.76 cm (10,911 BP).

8.5. Conclusions:

In conclusion, this study has demonstrated that a range of phenotypic and chemotaxonomic analyses is essential to differentiate and describe potentially new species assigned to the genera *Dermacoccus*, *Dietzia*, *Leifsonia* and *Rhodococcus*. Besides, genotypic analysis is also necessary to confidently differentiate closely related strains that have high similarities between each other, although nucleotide differences can be large. In addition, this study also provides evidence that the old freshwater sediments of Lake Suigetsu are comprised of potentially novel species. Further studies are also needed to determine whether strains F124T^{T}, F142T^{T}, F156T^{T}, F195T^{T}, F218T^{T}, F130T^{T} and F96T^{T} merit the recognition as new species.

Chapter 9 Concluding remarks and future work

In general, the bacterial communities' structure and diversity were affected by multiple environmental conditions in the sediments of Lake Suigetsu. The results have shown the potential of $\delta^{15}N$ isotope as an indicator for past climate change, however the $\delta^{15}N$ isotope may have also accounted for the nitrogen content from the nitrification or detoxification of ammonium (NH_4^+) in the sediments apart from the denitrification process. Therefore, the $\delta^{15}N$ isotope may be inappropriate as a correlation parameter to gauge the sediment nitrate content that was influenced by climatic change. Furthermore, in comparison to other investigated molecular techniques (eg. PCR-DGGE), metagenomics analysis is a promising approach which should be used to study the bacterial communities of the 18 sediments samples, so that the direct comparison between bacterial taxa and the environmental variables could further improve the search for potential microbial biomarkers for past climate change.

Based on the metagenomics approach, the evidence of seawater incursion on the Lake Suigetsu was discovered between 82.16 and 83.16 cm of the sediment depths, which is in proximity to the reported timeline on the historical records. It was found that the bacterial communities' structure and diversity (based on DGGE results) before and after the lake water transition (from fresh to brackish) were rather similar with minimal difference due to the changes in salinity. However, on a close inspection of the bacterial composition utilising the metagenomics analysis, distinctive differentiations were revealed between the pre and post salinity shift. The predominating taxa in freshwater sediments were different from those detected in the brackish sediments, indicating the likelihood of species replacement and/or cell inactivation due to the abrupt changes in water conditions. To better study the transition of salinity shift, further exploration of the bacterial community within sediment depths from 82.16 and 83.16 cm at a higher resolution should be carried out.

In addition, it was found that the direct impact of climatic temperature on bacterial communities was rather insignificant, instead the climate-dependent nutrient resources played a greater role in shaping the bacterial communities' structure and diversity. Based on metagenomics analysis, the taxa closely related to the colder climate were distinctive from the transition and warmer climatic conditions. This difference also

indicated that bacterial taxa that can tolerate and adapt to the changes of climatic temperature will be selected. During the transition of climate, *Xanthomonadaceae* from the phylum *Gammaproteobacteria* was found predominant in colder sediment deposits while *Caulobacteraceae* from the phylum *Alphaproteobacteria* were prevailing in warmer sediment deposits.

The application of combined techniques was evidently more effective in recovering a greater range of bacterial taxa, and molecular approaches could address a larger proportion of the bacterial community compared to culture-based techniques which were only limited to 'culturable' bacteria. Together, they could offer a more complete profile of bacterial diversity in Lake Suigetsu sediments. Furthermore, in the culture-dependent study, the comparison of freshwater taxa between PCR-DGGE and cultivation methods was less appropriate as the comparison could only be made at phylum level. To identify species at similar (genus) level, metagenomics sequencing would need to be employed to enhance the data to be more comparable.

For characterisation of the presumptively novel isolates, DNA-DNA hybridisation or whole-genome analysis is essential especially when the similarities between test strains and neighbouring type strains are higher than the threshold at 98.65%. Similarly, despite the wide variety of analyses that have been done, some of the chemotaxonomy analyses were not carried out due to the lack of time. Further work would be required, such as the identification of cellular sugars, menaquinones, diaminopimelic acid (DAP) and peptidoglycan structures. In addition, test strains that possessed 16S rRNA gene sequence lengths shorter than 1400 bp would need to be re-sequenced.

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Appendix 1: Dyes and solutions

a) PCR

Bromophenol blue (BPB; 6 × concentrate)

BPB was prepared at 6 × concentrate and diluted with the sample as required.

BPB 0.025 g, Sucrose 4.0 g, distilled H_2O to make up to 1 L

Tris-Acetate-EDTA buffer (TAE; 50 × concentrate stock solution)

Tris-HCL 242 g, 17.51 M glacial acetic acid 57.1 ml, 0.5 M EDTA (pH 8) 100 ml and distilled H_2O added to a final volume of 1 L. For 1× TAE, dilute 1 ml of 50 × stock solution with 50 ml of distilled H_2O.

Loading dye (6 × concentrate)

Sucrose 4 g, BPB 0.025 g and 10 ml 18.2 Ω MilliQ H_2O. The solution was kept at 4°C prior to use.

b) DGGE

Dcode Dye

BPB 0.05 g, Xylene cyanol 0.05 g, 1 × TAE 10 ml

Loading dye (2 × concentrate)

2% BPB 0.25 ml, 2% xylene cyanol 0.25 ml, 100% glycerol 7 ml and 18.2 Ω MilliQ H_2O 2.5 ml.

12 % acrylamide denaturing solution (per 100 ml)

All denaturing solutions were filter sterilized through a 0.45 µm filter and de-gassed for 30 minutes in the fume hood before storage at 4°C. For different denaturing %, add 2 ml of formamide and 2.1 g urea for every 5% of increment.

Denaturing solutions	**35%**	**55%**
40% (v/v) acrylamide (37.5:1 acrylamide:bisacrylamide)	30 ml	30 ml
50 × TAE	2 ml	2 ml
Deionised formamide	14 ml	22 ml
Urea (electrophoresis grade)	14.7 g	23.1 g
dH_2O	To 100 ml	To 100 ml

c) DDC

0.1% w/v Sodium cholate per 100 ml

Sodium cholate 0.1 g was added to 18. 2 Ω MilliQ H_2O 100 ml in a 100 ml Duran bottle. Solution was sterilized in autoclave machine at 121°C for 45 minutes prior use.

0.05 M Tris-Buffer (pH 7.4) per 100 ml

Tris-Base UltraClean 0.606 g was added to 18. 2 Ω MilliQ H_2O 100 ml in a 100 ml Duran bottle. Solution was autoclaved at 121°C for 45 minutes prior use.

¼ strength Ringer's solution

One Ringer's tablet (Oxoid, BR 52) was dissolved in 500 ml distilled H_2O to make 500 ml of ¼ strength Ringer's solution. The solution was autoclaved at 121°C for 45 minutes and kept at 4°C prior use.

d) Culture media

50 mg/ml Nystatin

Nystatin 0.5 g and sterilized 18. 2 Ω MilliQ H_2O 10 ml.

Store nystatin stock at -20°C.

20% v/v Glycerol solution

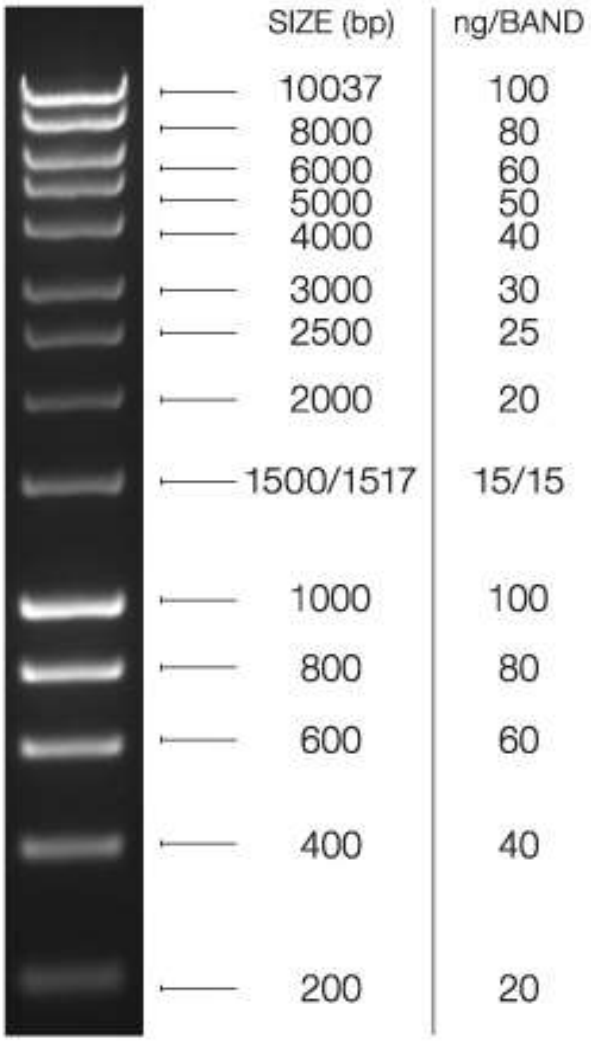
100% glycerol 100 ml and 18.2 Ω MilliQ H_2O 400 ml. The made up volume was allocated out in cap-tubings (1 ml) and sterilized in autoclave machine at 121°C for 45 minutes.

e) Bacterial DNA extraction

TE buffer (pH 8)

100 mM Tris-Cl (pH 8) 0.1211 g and 10 mM EDTA (pH 8) 0.0372 g in 10 ml of distilled H_2O

Appendix 2: Hyperladder I (Bioline)



Appendix 3: Culture media formulation (per L)

Trypticase Peptone Yeast Extract Agar (TPA; modified from Smith *et al*., 1982)
Tryptone 17 g, Peptone 3 g, Dextrose 2.5 g, Sodium chloride 5 g, Dipotassium phosphate 2.5 g, Yeast extracts 5 g, Agar 15 g; pH: 7.3

Autoclave at 121°C for 45 minutes, cool to 50°C and add 50 µg/ml of nystatin as a final concentration.
N.B: For purification of isolates, nystatin was not included. For broth, exclude agar from recipe.

Difco Marine Agar 2216 (MA)
Peptone 5 g, Yeast extract 1 g, Ferric citrate 0.1 g, Sodium chloride 19.45 g, Magnesium chloride 8.8 g, Sodium sulphate 2.24 g, Calcium chloride 1.8 g, Potassium chloride 0.55 g, Sodium bicarbonate 0.16 g, Potassium bromide 0.08 g, Strontium chloride 34 mg, Boric acid 22 mg, Sodium fluoride 2.4 mg, Ammonium nitrate 1.6 mg, Disodium phosphate 8 mg, Agar 15 g; pH 7.6 ± 0.2

Autoclave at 121°C for 45 minutes, cool to 50°C and add 50 µg/ml of nystatin as a final concentration.
N.B: For purification of isolates, nystatin was not included. For broth, exclude agar from recipe.

Nitrate minimal salt (NMS; Dworkin *et al*., 2006)
$MgSO_4.7H_2O$ 1 g, KNO_3 1 g, $Na_2HPO_4.12H_2O$ 0.717 g, KH_2PO_4 0.272 g, $CaCl_2.6H_2O$ 0.2 g, Ferric Ammonium citrate 5 mg, Trace element solution 1 ml; pH: 6.8

Trace Element Solution (per litre)
Disodium EDTA 0.5 g, $FeSO_4.7H_2O$ 0.2 g, H_3BO_3 0.03 g, $CoCl_2.6H_2O$ 0.02 g, $CuSO_4.5H_2O$ 0.03 g, $ZnSO_4.7H_2O$ 0.01 g, $MnCl_2.4H_2O$ 3 mg, $Na_2MoO_4.2H_2O$ 3 mg, $NiCl_2.6H_2O$ 2 mg; pH: 7

Autoclave at 121°C for 45 minutes, cool to 50°C and add 50 µg/ml of nystatin as a final concentration.

N.B: For purification of isolates, nystatin was not included. For broth, exclude agar from recipe.

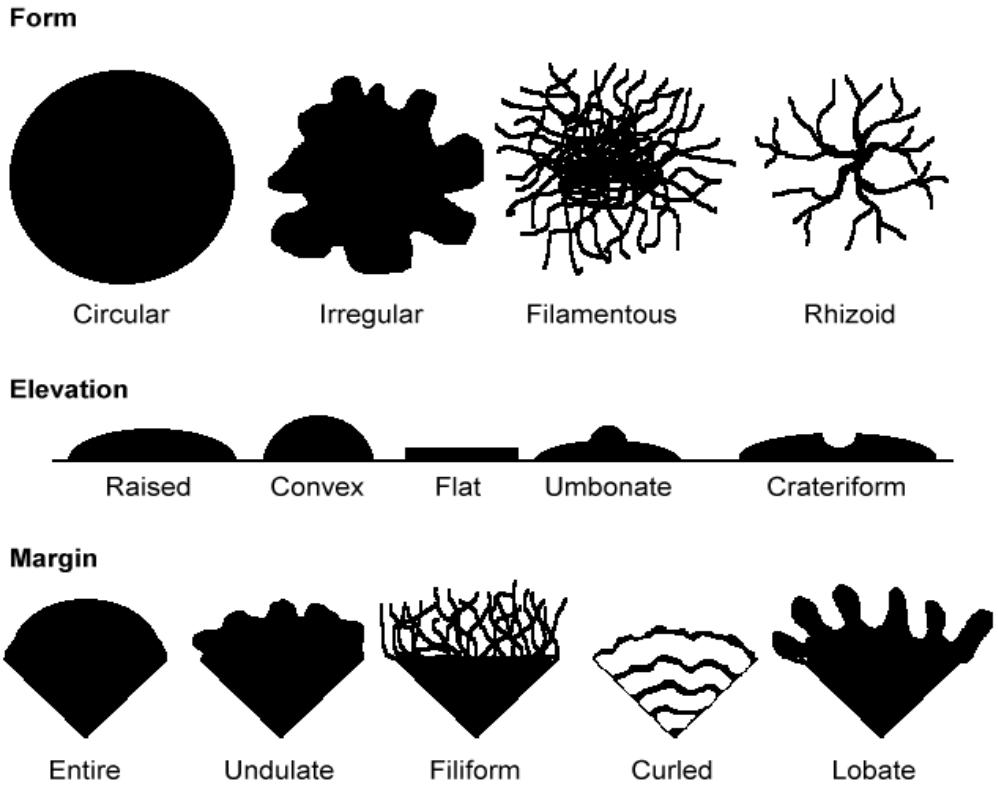
Artificial Sea Salt - Nitrate minimal salt (ASS-NMS; Dworkin *et al*., 2006)

NaCl 24.32 g, $MgCl_2$ 5.143 g, Na_2SO_4 4.06 g, $CaCl_2$ 1.14 g, KCl 0.69 g, $NaHCO_3$ 0.2 g, KBr 0.1 g, H_3BO_4 0.027 g, NaF 0.003 g, Na_2SiO_3 0.002 g, NH_4NO_3 0.002 g, NMS medium; pH: 7.5

Autoclave at 121°C for 45 minutes, cool to 50°C and add 50 µg/ml of nystatin as a final concentration.

N.B: For purification of isolates, nystatin was not included. For broth, exclude agar from recipe.

Appendix 4: Macro characterisation



Appendix 5: Table of 16S rRNA gene sequences of test strains

Code	Length (bp)	16S rRNA sequences
F124T	1423	GACGACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCT GGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTG GGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG CACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCA CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGG GCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAA GGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATT CCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGG ATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTC GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGGTG GGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGGTCTC AGTTCGGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAG TCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAA
F142T	1409	TGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCC CCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATATGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCT TGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAG AAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAA AGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG ATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTG GGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGAGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGT CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAAAGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGG AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTCGCGAAACCGTGAGGTGGAGCTAATCCCAAAAA ACCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC CCGTCAAGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACC
F156T	1421	ACGACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTG GGATAAGCCTGGGAAACTGGGTCTAATACTGGATATGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGG GGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC ACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCAC CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGG CTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAG GCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATTC CACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGA TTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGT GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAAAGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGG GGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGGTCTCA GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTC ACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAA

F116T	1420	ACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCT GGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTG GGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG CACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCA CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTCTGTCGCGTCTGCTGTGAAAGACCGGG GCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAA GGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATT CCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGG ATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTC GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGG GGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGGTCTCA GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTC ACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGA
F122T	1428	TGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCC CCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGC TTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAG AAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTYTGTCGCGTCTGCTGTGAA AGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG ATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTG GGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGT CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGA GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCTCAAAAAA CCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC CGTCAAGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGA
F170T	1441	AACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGG ATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGG TAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCT TAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGC AGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATTCCA CGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATT AATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTG TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGA TGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGGTCTCAGTT CGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACG AAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAAGTGGGACTGGCGATTGGGACTAA

1X X X

F218T	1535	AGCTCGATCCTCGCCTCAGGCTGGGTCACCTCCTTAGAGTTTGATCCTGGCTCAGGCTGGATCACCTCCTTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAA CACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGGATAAGCCTGGGAAACTGGGT CTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGAC GACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGG GTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTG ACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATTCCACGAGTTCCGTGCCGCAGCT AACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAA GAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA GTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCMTGGGAAACTGCCGGGGTCAACTCSGAAGAAGGTGGGGATGACGTCCAATCATCATGC CCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAAGGTTGCGAAACCGTGAGGTGGAACTAATCCCAAAAAACCGGTCTCAGTTCGGATTGGGGTCTGCAACT CGACCCCATGAAGTCGGARTCGCTAGTAATCGCAGATCAGCAACGCTGCCGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCGAA GCCGGTGGCCTAACCCTTGTGGGGGGARCCCTCCAAAGKGGGAMTGGSGATTTGGACT
F219T	1424	CAGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCA CTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTG GTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA TTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAA GCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACC GGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGC GAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACT CATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCAT GCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCA GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAG GTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGGT CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA AGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAA
F195T	1400	CCYCGCTCAGGCTTGATCACCTCCTTWGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCGGGGTACACGAGCGG CGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCCTATCGCATGGTGGGTGGTGGAAAGATTTA TCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACAC GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACC AGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGA GCTTGTAGGCGGTCTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCG GTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTA GTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA AGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTC TTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACCCGCAGCTAAC GCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAA CCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC CCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCAC

F72T	1449	TCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTC ACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTT GGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT ATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGA AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTCTGTCGCGTCTGCTGTGAAAGAC CGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGG CGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGAC TCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCA TGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTC AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAA GGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGAGGTGGAGCTAATCCCAAAAAACCGG TCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA AGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAAGTGGGACTGGCGATTGGGACTAA
F130T	1344	AACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTAAGGCCCTTTCGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGGGTAATCTGCCCTGCACTTCGGGATA AGCCTGGGAAACCGGGTCTAATACCGGATATGAGCTCCTGCCGCATGGTGGGGGTTGGAAAGTTTTTCGGTGCAGGATGAGTCCGCGGCCTATCAGCTTGTTGGTGGGGTAATG GCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG GCGAAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGTCTTCGGATTGTAAACTCCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAA CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCACGTCGTCTGTGAAATCCTCCAGCTCAACT GGGGGCGTGCAGGCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGT CTCTGGGTAGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGCGCTAGGTGTGGGGTCCTTCCACGGA TTCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATT CGATGCAACGCGAAGAACCTTACCTAGGCTTGACATATACAGGACGACGGCAGAGATGTCGTTTCCCTTGTGGCTTGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCTCATGTTGCCAGCACGTTATGGTGGGGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATG ACGTCAAATCATCATGCCCCTTATGTCTAGGGCTTCACACATGCTACAATGGCTAGTACAGAGGGCTGCGAGACCGCGAGGTGGAGCGAATCCCTTAAAGCTAGTCTCAGTTCG GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
F83T	1545	TGGATCACCTCCTTWGWGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTGGAGCTTGCTCTAGGGGATTAGTGGCGAAC GGGTGAGTAACACGTGAGTAACCTGCCCTTGACTCTGGGATAACCTCCGGAAACGGAAGCTAATACCGGATATGACGTACGGAGGCATCTCCTGTACGTGGAAAGAACTTCGGT CAAGGATGGACTCGCGGCCTATCAGGTAGTTGGTGAGGTAACGGCCCACCAAGCCTACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGA AGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCG TAGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGG AATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA CGCCGTAAACGTTGGGCGCTAGATGTGGGGACCATTCCACGGTTTCCGTGTCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGGCCAGAAATGGTCAACTCTTTGG ACACTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAAC TCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAA TACCGTAAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGA ATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAAGGTGGGATCGGTGATTAG GACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCATAGAGTTTGATCCTGG

F96T	1531	TGGATCACCTCCTTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTGGAGCTTGCTCTAGGGGATTAGTGGCGAACG GGTGAGTAACACGTGAGTAACCTGCCCTTGACTCTGGGATAACCTCCGGAAACGGAAGCTAATACCGGATATGACGTACGGAGGCATCTCCTGTACGTGGAAAGAACTTCGGTC AAGGATGGACTCGCGGCCTATCAGGTAGTTGGTGAGGTAACGGCCCACCAAGCCTACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCA GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAA GAAGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGT AGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGA ATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGTCTCTGGGCCGTAACTGACGCTGAGGAGCTAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTNCACG CCGTAAACGTTGGGCGCTAGATGTGGGGACCATTCCACGGTTTCCGTGTCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATT GACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGGCCAGAAATGGTCAACTCTTTGGAC ACTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTC ATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATA CCGTAAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAAT ACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAAGGTGGGATCGGTGATTAGGA CTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCATAG
F71T	1451	TCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTGGAGCTTGCTCTAGGGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTG ACTCTGGGATAACCTCCGGAAACGGAAGCTAATACCGGATATGACGTACGGAGGCATCTCCTGTACGTGGAAAGAACTTCGGTCAAGGATGGACTCGCGGCCTATCAGGTAGTT GGTGAGGTAACGGCCCACCAAGCCTACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT ATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAA AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACC CGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGAATGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGG CGAAGGCAGTTCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGATGTGGGGA CCATTCCACGGTTTCCGTGTCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCA TGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGGCCAGAAATGGTCAACTCTTTGGACACTCGTAAACAGGTGGTGCATGGTTGTCG TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATAGGAGACTGCCGGGGTCAACTCGGAG GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTAAGGTGGAGCGAATCCCAAAAAGC CGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC GTCAAGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAAGTGGGATCGGTGATTAGGACTAA
F42M	1547	TTGATCTCGTCTCTKGCTGGATCACCTCCTTAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGT GGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGT GGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGG GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA CCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACT GGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTC CTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAG ATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTCCTTCCACGGAATCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGC TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATACCGGAAAGCTGCAGAGA TGTGGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATGTTGCCAGCAC GTTATGGTGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCAGT ACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGCTGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAG CAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGACCCGTCAAAGGkG GGACTGGCAATTGGGACTAATTCTAAACAAGGTACCCGTACCGAAAGGTGCGGCTGGATCACCTCCTTA

F152M	1252	GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTTCGGG ATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCCTATCGCATGGTGGGTGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGT AATGGCCTACCAAGGCGAGGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACTGGAGGCAGCAGTGGGGAATATTGCACA ATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGG CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTC AACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCG GGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGSGCTAGGTGTGGGTTCCTTCCACG GAATCCGTGCCATATCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGGGGATCATGTGGATTAA TTCAATGCAACGCGAAAAACCTTACCTGGGTTTGACATATACCGAAAACCTGCAAAAATGTGGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCCTCAGCTCGTGTC CTGAGATGTTGGGTTAAATCCCGCAACCAGCGCAACCCCTATCTTATGTTGCCAGCACGTTATGGTGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAC GACGTCAAATCATCATGCCCCTTATGTCCTGGGCTTCACACATGCTACAATGGCCAGTACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGCTGGTCTCAGTTC GGA
F39T	1412	TGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCAC TTCGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCCTATCGCATGGTGGGTGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGG TGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT GCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGC ACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGC AGCTCAACTGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGA AGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTCCT TCCACGGAATCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTG GATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATACCGGAAAGCTGCAGAGATGTGGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATGTTGCCAGCACGTTATGGTGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGG TGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCAGTACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGCTGGTC TCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC GTCATGAAAGTCGGTAACACCCGAAGCCCGGTGGCTTAACCCCTTGTGGG
F31M	1415	CGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGGTAAGGCCTTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCCTGCACTTCGGGAT AAGCCTGGGAAACTGGGTCTAATACCGGATATGACCTCCTATCGCATGGTGGGTGGTGGAAAGATTTATCGGTGCAGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAA TGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTA ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAAC TGCTGGCTTGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGT CTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTCCTTCCACGGA ATCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATT CGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATACCGGAAAGCTGCAGAGATGTGGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATGTTGCCAGCACGTTATGGTGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACG ACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCAGTACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGCTGGTCTCAGTTCG GATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAA AGTCGGTAACACCCGAAGCCGGTGGCTTAACCCCTTGTGGGAGGAGCCGTCGA

XXXV

Appendix 6: Table of source and information of type strains

DSM 45334	*Dietzia aerolata*	Kämpfer *et al*. (2010)
DSM 45698	*Dietzia alimentaria*	Kim *et al*. (2011)
DSM 45140	*Dietzia cercidiphylli*	Li *et al*. (2008b)
DSM 44904	*Dietzia cinnamea*	Yassin *et al*. (2006)
DSM 44748	*Dietzia dagingensis*	Nazina *et al*. (Unpublished data)
DSM 44907	*Dietzia kunjanmensis*	Mayilraj *et al*. (2006)
DSM 45074	*Dietzia lutea*	Li *et al*. (2009)
DSM 43672	*Dietzia maris*	Rainey *et al*. (1995)
DSM 44198	*Dietzia natronolimnaea*	Duckworth *et al*. (1998)
DSM 44820	*Dietzia psychralcaliphila*	Yumoto *et al*. (2002)
DSM 45139	*Dietzia schimae*	Li *et al*. (2008b)
DSM 17573	*Dermacoccus abyssi*	Pathom-aree *et al*. (2006a)
DSM 20448	*Dermacoccus nishinomiyaensis*	Stackebrandt *et al*. (1995)
DSM 17574, DSM 17575	*Dermacoccus barathri*, *Dermacoccus profundi*	Pathom-aree *et al*. (2006b)
DSM 15202	*Leifsonia poae*	Evtushenko *et al*. (2000)
DSM 23871	*Leifsonia soli*	Madhaiyan *et al*. (2010)
DSM 15165, DSM 15166	*Leifsonia shinshuensis*, *Leifsonia naganoensis*	Suzuki *et al*. (1999)
DSM 45380	*Rhodococcus artemisiae*	Zhao *et al*. (2012)
DSM 45608	*Rhodococcus nanhaiensis*	Li *et al*. (2012)
DSM 45257	*Rhodococcus jialingiae*	Wang *et al*. (2010)
DSM 44587	*Rhodococcus baikonurensis*	Li *et al*. (2004)
KCTC 19205	*Rhodococcus qingshengii*	Xu *et al*. (2007)
DSM 43066	*Rhodococcus erythropolis*	Yoon *et al*. (1997)
DSM 43954	*Rhodococcus globerulus*	Goodfellow *et al*. (1982)
DSM 45141	*Rhodococcus cercidiphylli*	Li *et al*. (2008a)
CCM 4760	*Rhodococcus jostii*	Takeuchi *et al*. (2002)

Appendix 7: Culture media formula for phenotypic characterisation

a) Culture media

Aesculin and Arbutin degradation (Williams *et al*., 1983)

Basal medium: Arbutin 1 g, Ferric ammonium citrate 5 g, Peptone 10 g, Sodium chloride 1 g and Agar 15 g; pH 7.2.

Test medium: as for basal medium but supplemented with aesculin (1.0 g) or arbutin (1.0 g). The molten media was dispensed aseptically in 20 ml into Petri dish.

1% Allantoin reduction (Korn-Wendisch and Schneider 1992)

Glucose 1 g, peptone 1 g, sodium dihydrogen phosphate 1.98 g, potassium dihydrogen phosphate 1.51 g, magnesium sulphate 0.5 g, sodium chloride 5 g, phenol red 0.012 g, agar 12 g, distilled H_2O 1 L, pH 6.8. Once sterilized the tyndallised allantoin (10 g) was added. The media were dispensed aseptically in 3 ml amounts into sterile bijou and allowed to set as a slope.

Buffered media for pH tolerance test

Buffered media (pH 4.0 to 4.5)

Buffers were prepared at pH 4.0 and 4.5 by dissolving the following components in 100 ml of distilled H_2O and sterilizing by autoclaving at 121°C for 15 minutes. Each buffer was added aseptically to 100 ml of sterile cooled double strength GYEA medium to give a final volume of 200 ml.

Final pH of medium	Citric acid (g/100 ml)	Na_2HPO_4 (g/100 ml)	KCl (g/100 ml)
4.0	2.58	5.52	5.08
4.5	2.24	6.68	4.32

Buffered media (pH 5.0 to 9.0)

Buffer A: 15.6g sodium dihydrogen phosphate dissolved in 500 ml of distilled H_2O.

Buffer B: 14.195g *di*-sodium hydrogen phosphate dissolved in 500 ml of distilled H_2O.

Phosphate buffers were prepared in the range pH 5.0 to 9.0 by combining the following volumes if buffers A and B and sterilising by autoclaving at 121°C for 15 minutes. Each

buffer was added aseptically to 100 ml of sterile cooled double strength GYEA medium to give a final volume of 200 ml.

Final pH of medium	Buffer A (ml)	Buffer B (ml)
5.0	99	1
5.5	97	3
6.0	87.7	12.3
6.5	68	32
7.0	39	61
7.5	16	84
8.0	5.3	94.7
8.5	2.5	97.5
9.0	1	99

Buffered media (pH 9.5 to 10.0)

Solution A: 14.31g *di*-sodium carbonate (Na_2CO_3) dissolved in 500 ml of distilled H2O
Solution B: 4.205g sodium hydrogen carbonate ($NaHCO_3$) dissolved in 500 ml of distilled H_2O.

Buffers were prepared at pH 4.0 and 4.5 by combining the following volumes of solutions A and B and sterilizing by autoclaving at 121°C for 15 minutes. Each buffer was added aseptically to 100 m of sterile cooled double strength GYEA medium to give a final volume of 200 ml.

Final pH of medium	Buffer A (ml)	Buffer B (ml)
9.5	40	70
10.0	60	30

Gelatin degradation (Gordon, 1967)

Gelatin 4 g, Lab Lemco 2.4 g, peptone 5 g, agar 12 g; pH 7.0.

Glucose yeast extract agar (GYEA; Gordon and Mihm, 1962)

Glucose 10 g, yeast extract 10 g, agar 12 g; pH 7.2.

Glycerol broth supplemented with 0.0005% (w/v) lysozyme

Glycerol broth supplemented with 0.005% (w/v) lysozyme was prepared by dissolving 0.1 g of lysozyme in 100 ml distilled H_2O in a volumetric flask and sterilising the resultant solution by filtration. A 15 ml amount of the lysozyme solution was mixed with 285 ml of sterile glycerol broth (made up with peptone 5 g, beef extract 3 g,

glycerol 70 ml, distilled H_2O 1 L; pH 7.0). The medium was dispensed aseptically in 2.5 ml amounts into sterile bijou.

Indole test medium (Lányi, 1987)

Bacto peptone 10 g, NaCl 5g; pH 7.2-7.4

Autoclaved broth at 121°C and dispense 3 ml in sterile bijou tubes.

Methy red (MR) & Voges-Proskauer (VP) test medium (Lányi, 1987)

Bacto peptone 7 g, K_2PHO_4 5g, glucose 5g; pH 7.5

Autoclaved broth at 121°C and dispense 3 ml in sterile bijou tubes.

Nitrate broth (Gordon, 1967)

Potassium nitrate 1 g, peptone 5 g, Lab Lemco 2.4 g; pH 7.4. The medium was dispensed aseptically in 3 ml aliquots into sterile plastic bijous.

Reagent A: 0.8 g sulfanilic acid in 100 ml of 5 M acetic acid.

Reagent B: 0.6 g 8-amino-2-naphthelene-2-sulphuric acid

RNA

RNA 3 g, sodium chloride 5 g, tryptone 20 g, agar 15 g; pH 7.3

Sierra's medium (Sierra, 1957)

Bacto-peptone 10 g, calcium carbonate 0.1 g, sodium chloride 5 g, agar 12; pH 7.4

Stevenson's medium (Stevenson, 1967)

Stevenson's basal medium was prepared to give a final volume of 200 ml, as follows;

1. Yeast Nitrogen Base (Difco), 1.34 g dissolved in 20 ml of distilled H_2O and supplemented with 2 mg of Casamino acids (Difco). The solution was sterilised by membrane filtration.

2. A 40 ml volume of 1% w/v di-potassium hydrogen phosphate was prepared and sterilised by autoclaving at 121°C for 15 minutes.

3. 2.4 g agar in 130 ml distilled H_2O was steamed until dissolved, sterilized by autoclaving at 121°C for 15 minutes and cooled to 55°C.

4. Concentrated solutions (× 40) of the various carbon source compounds were prepared in 10 mlo distilled H_2O and sterilisd by membrane filtration or Tyndallisation at 100°C for 30 minutes on three consecutive days.

The sterile, cooled solutions were combined aseptically to provide a final carbon source concentration of 1.0%, w/v or 0.1%, w/v. A positive control medium was prepared as above with 1%, w/v glucose as the sole carbon source. A negative control medium was prepared as above but without the addition of a carbon source.

Tributyrin agar

Tributyrin agar base (20 g; Sigma-Aldrich T3688) was dissolved in 190 ml distilled H_2O by heating and stirring on a heated magnetic stirrer. Tributyrin (10 ml; Sigma T8626) was added into the medium while it was being stirred. The medium was sterilized by autoclaving at 121°C for 15 minutes, cooled at 50°C in H_2O bath and mixed gently by swirling while pouring in order to maintain uniform turbidity in the agar medium.

Urea reduction (Korn-Wendisch and Schneider 1992)

Glucose 1 g, monopotassium phosphate 2 g, peptone 1 g, sodium chloride 5 g, phenol red 0.012 g, agar 15 g, distilled H_2O 1 L; pH 6.8. Once sterilized the 5 ml of filter sterilized 40% urea solution was added. The media were dispensed aseptically in 3 ml amounts into sterile bijou and allowed to set as a slope.

Degradation tests (per 200 ml)

0.4% Adenine 0.8 g; 0.1% Aesculin 0.2 g; 1% Allantoin 2 g; 0.1% Arbutin 0.2 g; 0.1% Casein 0.2 g; 1% Cellulose 2 g; 0.3% DNA 0.6 g; 0.3% Elastin 0.6 g; 0.4% Gelatin 0.8 g; 0.5% Guanine 1 g; 0.4% Hypoxanthine 0.8 g; 0.3% RNA 0.6 g; 0.1% Starch 0.2 g; 1% Tributyrin 2 g; 1% Tween 20, 40, 60 2 g; 0.5% L-tyrosine 1 g; 0.2% Urea 0.4 g; 0.5% Uric acid 1 g; 0.4% Xanthine 0.8 g; 0.4% Xylan 0.8 g were suspended in distilled H_2O (100 ml) and sterilised by tyndallisation at 121°C for 15 minutes. These compounds were then thoroughly mixed with 190 ml of sterile molten GYEA to give the appropriate final concentration and dispensed into sterile petri dishes. Casein (0.2 g) was directly added to a 200 ml of GYEA and autoclaved.

Acid production from sugars (Gordon *et al*., 1974)

Basal medium: diammonium hydrogen phosphate 1 g, potassium chloride 0.2 g, magnesium sulphate 0.2 g, agar 15 g; pH 7.0. 15 ml of sterile aqueous solution of bromocresol purple (0.04%, w/v) was added after sterilization.

Carbohydrate solutions: these solutions (10%, w/v) were prepared, separately autoclaved then individually mixed thoroughly with molten basal medium agar to give a final concentration of 1% (w/v) before pouring.

b) Reagents and solutions

Aqueous methanol

0.3% w/v aqueous sodium chloride 10 ml in 100 ml of methanol

5% ethanolic molybdophosphoric acid

Molybdophosphoric acid 5 g; Absolute ethanol 100 ml

20% v/v H_2O_2 solution for catalase production test:

10 ml of 100% of H_2O_2 was diluted in 50 ml distilled H_2O to yield 20% v/v H_2O_2 solution.

Lead acetate strips

Lead acetate strips (Whatman, Maidstone, UK) were cut into 3 cm long strips, folded at one end and autoclaved in a glass petri dish.

Lugol's iodine solution (Cowan and Steel, 1974)

Iodine 5 g, potassium iodine 10 g, distilled H_2O 1 L. The compounds were dissolved initially in 10 ml of distilled H_2O and the volume then made up to 100 ml. Prior to use the reagent was diluted 1 in 5 with distilled H_2O .

Kovács's reagent for indole test

Solution A: *p*-dimethylaminobenzaldehyde 5 g, Isoamyl alcohol 75 ml

Dissolve by gently heating in water bath and store at 4°C in a refrigerator.

Solution B: concentrated HCl at analytical grade

:. 3 volumes of Solution A + 1 volume of Solution B = Kovács's reagent

5.0 McFarland standard: (McFarland, 1907)

1% Barium chloride 5 ml, 1% sulphuric acid 95 ml

1.5% v/v of methanolic sulphuric acid

Concentrated sulphuric acid 1.5 ml in 98.5 ml of anhydrous methanol

Methyl red reagent

Methyl red 0.25 g, ethanol 100 ml

Add one drop of MR reagent to test.

Oxidase test

0.5 g of N, N-tetramethyl-p-phenylenediamine dihydrochloride in 50 ml of distilled H_2O.

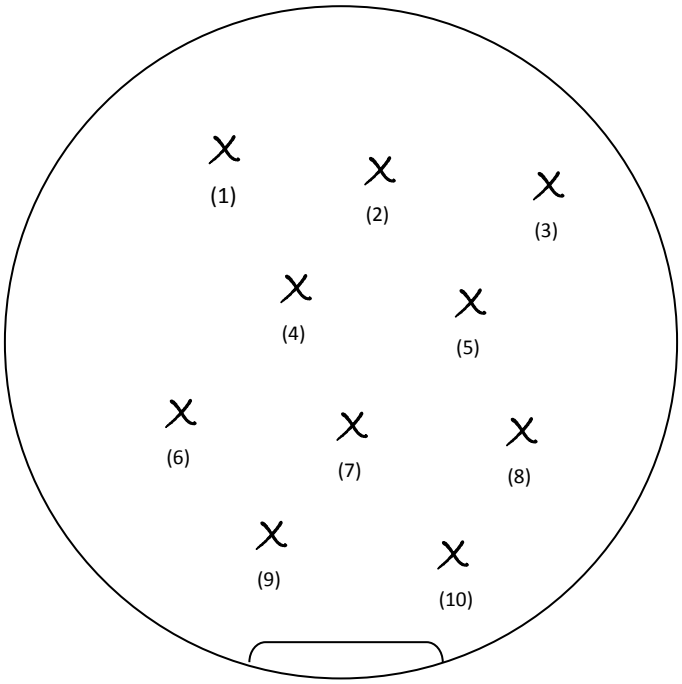
Voges-Proskauer (VP) test reagents (Lányi, 1987)

Reagent A: α-Naphthol 5g, absolute ethanol 100 ml (the reagent must be darker than straw colour)

Reagent B: Potassium hydroxide 40g, distilled H_2O 100 ml

Add 0.6 ml Reagent A and 0.2 ml Reagent B per ml of culture to test.

Appendix 8: Inoculum template



Appendix 9: Degradation tests- incubation periods and tests methods

Adenine

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which is a positive result.

Aesculin

Incubate culture plates at 28°C and examine at 7 and 14 days. Compare growth to control plate (with no aesculin) and a positive score when medium become blackened.

Allantoin

Incubate bijou tubes at 28°C and examine at 7 and 14 days for positive result, which medium colour will change from orange to pink-red, indicating a positive alkaline reaction.

Arbutin

Incubate culture plates at 28°C and examine at 7 and 14 days by comparing to the control plate (with no arbutin). Blackened medium indicates a positive test.

Casein

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which is a positive result.

Cellulose

Incubate culture plates at 28°C and examine at 7, 14 and 21 days. Flood the culture plates with 0.1% (w/v) Congo red for 15 minutes at room temperature. Remove any excess reagent and a pale orange to straw colour zone around the growth areas will be a positive result.

DNA

Incubate culture plates at 28°C for 7 days. Flood the growth cultures with 1M HCl solution and the presence of halos is a positive test.

Elastin

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which indicates a positive result.

Gelatin

Incubate culture plates at 28°C for 7 days. Flood the growth cultures with 30% (w/v) trichloroacetic acid (TCA) and a clearing zone under and around the growth areas represents a positive score.

Guanine

Incubate culture plates at 28°C and examine at 7, 14, 21, 28 and 35 days for the presence of halos which represents a positive score.

Hypoxanthine

Incubate culture plates at 28°C and examine at 7, 14 and 21 days and a positive result when the presence of halos develops under and around the growth areas.

L-tyrosine

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which represents a positive result.

RNA

Incubate culture plates at 28°C for 7 days. Flood the growth cultures with 1M HCl solution and the presence of halos indicates a positive score.

Starch

Incubate culture plates at 28°C and examine at 7, 14 and 21 days. Flood culture plates with Lugol's iodine solution (Appendix 7) and a clearing zone under and around the area of growth represent a positive result.

Tributyrin

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which indicates a positive result.

Tween 20, 40 and 60

Incubate culture plates at 28°C and examine at 7 and 14 days for precipitation of calcium salts of fatty acids under and around areas of growth, which indicates a positive result.

Urea

Incubate bijou tubes at 28°C and examine at 7 and 14 days for the development of an alkaline reaction that changes medium colour from orange to pink-red, in which this implies a positive result.

Uric acid

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which will be a positive result.

Xanthine

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which is a positive result.

Xylan

Incubate culture plates at 28°C and examine at 7, 14 and 21 days for the presence of halos which indicates a positive result.

Appendix 10: Table of unit characters scored in the phenotypic characterisation of the test strains

Unit character

Biochemical tests (%, w/v):
Catalase reactions
H_2S production
Indole production
Methyl red test
Nitrate reduction (0.1)
Nitrite reduction (0.1)
Oxidase test
Voges–Proskauer test
Lysozyme resistance (0.1)

Degradation tests (%, w/v):
Adenine (0.4)
Aesculin (0.1)
Allantoin (1.0)
Arbutin (0.1)
Casein (0.1)
Cellulose (1.0)
DNA (0.3)
Elastin (0.3)
Gelatin (0.4)
Guanine (0.5)
Hypoxanthine (0.4)
RNA (0.3)
Starch (0.1)
Tributyrin (1.0)
Tween 20 (1.0)
Tween 40 (1.0)
Tween 60 (1.0)
L-Tyrosine (0.5)
Urea (0.2)
Uric acid (0.5)
Xanthine (0.4)
Xylan (0.4)

Morphological tests:
Growth on glucose-yeast extract agar:
Colony colour (light/strong orange,
Form (circular/irregular)
Elevation (raised/convex/umbonate)
Margin (entire/undulate)
Colony diameter in cm

Enzymatic reaction tests using APIzyme:
Alkaline phosphatase
Esterase (C4)
Esterase Lipase (C8)
Lipase (C14)
Leucine arylamidase
Valine arylamidase
Cystine arylamidase
Trypsin
α-chymotrypsin
Acid phosphatase
α-galactosidase
β-galactosidase
Naphthol-AS-BI-phosphohydrolysase

Unit character

β-glucuronidase
α-glucosidase
β-glucosidase
N-acetyl-β-glucosaminidase
α-mannosidase
α-fucosidase

Acid productions from sugar (0.5%, w/v):
Acetamide
Adonitol
D-(-)-arabinose
D-(-)-salicin
D-(+)-mannose
D-(+)-xylose
D-cellobiose
D-fructose
D-galactose
D-glucose
D-mannitol
D-raffinose
D-ribose
D-sorbitol
Dulcitol
Glycerol
Inulin
Lactose
L-arabinose
Maltose
Melibiose
meso-erythritol
myo-inositol
Starch
Sucrose
Trehalose
α-L-rhamnose

Growth on sole carbon sources (1%, w/v):
1,2-propanediol
2,3-butanediol
4-aminobutyrate
Adonitol
Aesculin
Amygdalin
Arbutin
Butane-1,3-diol
Butane-1-ol
Butane-1,4-diol
Casein
Cellulose
D-alanine
D-arabinose
D-arabitol
Dextrin
D-fructose
D-galactose
D-glucosamine HCl
D-cellobiose

Unit character

Growth on sole carbon sources (1%, w/v):

D-glucose
D-mannitol
D-melezitose
D-melibiose
D-raffinose
D-ribose
D(-)salicin
D-sorbitol
Dulcitol
D-xylose
Elastin
Ethanol
Glycerol
Glycogen
Guanine
Hypoxanthine
Inulin
Iso-amyl alcohol
Lactose
L-alanine
L-arabinose
L-arabitol
L-sorbose
L-xylose
Maltose
meso-erythritol
myo-inositol
Methyl caprate
Methyl α-D-glucopyranoside
Methyl α-D-mannopyranoside
Methyl β-D-xylopyranoside
N-Acetyl D-glucosamine
Starch
Sucrose
Trehalose
Tween 40
Tyramine
Xanthine

Growth on sole carbon sources (0.1%, w/v):

Acetic acid
Adipic acid
Anthranilic acid
Betaine
D-galacturonic acid
D-glucuronic acid
D-mandelic acid
Fumaric acid
D-gluconic acid
Glutaric acid
DL-lactic acid
L-malic acid
m-hydroxybenzoic acid
Paraffin
p-Hydroxybenzoic acid
Pimelic acid
Sebacic acid
Sodium acetate
Sodium benzoate

Unit character

Sodium citrate
Sodium formate
Sodium glutamate
Sodium malate
Sodium malonate
Sodium oleate
Sodium oxalate
Sodium propionate
Sodium pyruvate
Sodium succinate
Sodium tartrate
Spermine
Valeric acid
Vanillin

Growth on sole nitrogen sources (0.1%, w/v):

Acetamide
Benzamide
D-alanine
D-glucosamine HCl
DL-methionine
DL-norleucine
DL-norvaline
Ethanolamine
Gelatin
Glycine
L-alanine
L-arginine
L-asparagine
L-aspartic acid
L-cysteine
L-glutamic acid
L-glutamine
L-histidine
L-leucine
L-lysine
L-ornithine
L-phenylalanine
L-proline
L-serine
L-threonine
L-tryptophan
L-tyrosine
L-valine
Potassium nitrate
Urea

Inoculum determined using McFarland standard at 5.0

Appendix 11:

Table of Raup-Crick Similarity index-analysis of bacterial communities across climatic changed (part 1)

	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101
56		1.00	1.00	0.83	0.89	0.39	0.60	0.46	0.33	0.59	0.49	0.19	0.92	0.75	0.81	0.67	0.27	0.28	0.22	0.07	0.05	0.01	0.06	0.21	0.12	0.10	0.15	0.21	0.49	0.10	0.24	0.80	0.18	0.53	0.33	0.28	0.13	0.32	0.46	0.50	0.59	0.27	0.35	0.47	0.33	0.29
57			0.99	0.92	0.97	0.61	0.76	0.86	0.52	0.77	0.37	0.87	0.98	0.85	0.98	0.81	0.67	0.69	0.35	0.15	0.32	0.14	0.32	0.32	0.23	0.30	0.06	0.23	0.32	0.29	0.05	0.09	0.26	0.58	0.46	0.46	0.22	0.48	0.49	0.62	0.84	0.18	0.44	0.60	0.77	0.70
58				0.96	1.00	0.55	0.45	0.42	0.03	0.27	0.01	0.06	0.32	0.06	0.50	0.50	0.11	0.54	0.18	0.43	0.58	0.08	0.02	0.05	0.42	0.36	0.25	0.20	0.36	0.45	0.03	0.14	0.05	0.45	0.03	0.14	0.05	0.19	0.02	0.08	0.41	0.18	0.26	0.04	0.38	0.42	0.43	0.26
59					1.00	0.93	0.73	0.84	0.52	0.72	0.64	0.68	0.72	0.40	0.77	0.34	0.47	0.76	0.88	0.64	0.79	0.33	0.32	0.65	0.76	0.45	0.67	0.70	0.45	0.49	0.59	0.46	0.83	0.86	0.79	0.88	0.92	0.66	0.77	0.66	0.88	0.54	0.74	0.21	0.95	0.96	0.92	0.99
60						0.97	0.71	0.94	0.70	0.45	0.21	0.56	0.51	0.12	0.39	0.24	0.18	0.42	0.31	0.21	0.14	0.03	0.04	0.33	0.60	0.18	0.30	0.33	0.66	0.46	0.23	0.04	0.25	0.38	0.79	0.80	0.80	0.97	0.86	0.10	0.74	0.33	0.54	0.22	0.25	0.79	0.80	0.80	0.97
61							0.96	0.95	0.85	0.24	0.38	0.50	0.48	0.51	0.32	0.41	0.12	0.32	0.24	0.17	0.13	0.11	0.12	0.25	0.13	0.40	0.25	0.94	0.72	0.86	0.25	0.16	0.03	0.17	0.62	0.12	0.49	0.88	0.74	0.59	0.47	0.43	0.49	0.80	0.81	
62								0.99	0.98	0.95	0.79	0.88	0.42	0.69	0.29	0.35	0.27	0.26	0.19	0.01	0.70	0.21	0.26	0.68	0.10	0.40	0.15	0.43	0.09	0.07	0.46	0.03	0.01	0.16	0.13	0.03	0.97	0.06	0.72	0.66	0.36	0.43	0.25	0.28			
63									1.00	0.97	0.88	0.93	0.93	0.77	0.86	0.87	0.62	0.65	0.29	0.09	0.41	0.83	0.79	0.26	0.83	0.79	0.15	0.54	0.89	0.09	0.28	0.46	0.03	0.79	0.29	0.03	0.20	0.87	0.78	0.91	0.78	0.91	0.93	0.84	0.87			
64										1.00	1.00	0.99	0.84	0.88	0.76	0.83	0.93	0.26	0.43	0.04	0.70	0.86	0.88	0.76	0.14	0.69	0.48	0.08	0.82	0.84	0.43	0.36	0.90	0.89	0.97	0.81	0.96	0.97	0.73	0.91								
65											1.00	0.97	0.95	0.68	0.92	0.82	0.92	0.26	0.68	0.13	0.96	0.79	0.88	0.92	0.96	0.06	0.20	0.42	0.58	0.75	0.89	0.75	0.91	0.88	0.99	0.94	0.85	0.87	0.51	0.78										
66												0.98	0.98	0.93	0.87	0.45	0.85	0.14	0.54	0.25	0.94	0.57	0.84	0.94	0.26	0.26	0.01	0.47	0.76	0.99	0.36	0.88	0.39	0.65	0.80	0.93	0.76	0.84	0.66											
67													0.83	0.56	0.87	0.49	0.83	0.86	0.81	0.55	0.74	0.49	0.26	0.26	0.41	0.18	0.04	0.53	0.05	0.76	0.03	0.41	0.84	0.99	0.88	0.80	0.94	0.68	0.15	0.98	0.93	0.87	0.97								
68														1.00	1.00	0.92	0.89	0.69	0.60	0.32	0.26	0.96	0.47	0.83	0.71	0.79	0.32	0.60	0.71	0.15	0.61	0.20	0.79	0.62	0.52	0.78	0.70	0.96	0.34	0.96	0.85	0.63	0.93	0.95	0.96	0.97	

Table of Raup-Crick Similarity index-analysis of bacterial communities across climatic changed (part 2)

82	81	80	79	78	77	76	75	74	73	72	71	70	
													56
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													66
													67
													68
													69
													70
												1.00	**71**
											1.00	1.00	**72**
										1.00	1.00	0.98	**73**
									0.96	0.85	0.49	0.34	**74**
								1.00	0.88	0.66	0.22	0.36	**75**
							0.99	0.98	0.57	0.80	0.17	0.31	**76**
						0.99	0.93	0.74	0.84	0.95	0.86	0.85	**77**
					1.00	0.91	0.17	0.49	0.80	0.99	0.96	0.83	**78**
				1.00	0.98	0.90	0.29	0.81	0.87	1.00	0.91	0.85	**79**
			0.68	0.13	0.54	0.03	0.05	0.04	0.23	0.75	0.35	0.75	**80**
		0.99	0.37	0.07	0.13	0.02	0.01	0.00	0.08	0.23	0.10	0.47	**81**
	0.97	0.98	0.77	0.23	0.33	0.51	0.09	0.20	0.05	0.23	0.15	0.23	**82**
1.00	0.99	0.87	0.81	0.47	0.75	0.74	0.30	0.25	0.12	0.33	0.21	0.33	**83**
0.93	0.78	0.93	0.85	0.63	0.46	0.35	0.35	0.24	0.29	0.61	0.50	0.61	**84**
0.62	0.79	0.45	0.34	0.72	0.22	0.71	0.53	0.12	0.19	0.19	0.26	0.18	**85**
0.48	0.17	0.88	0.28	0.08	0.51	0.23	0.94	0.56	0.12	0.63	0.23	0.63	**86**
0.44	0.34	0.73	0.05	0.03	0.08	0.03	0.55	0.42	0.52	0.10	0.14	0.10	**87**
0.91	0.75	0.95	0.49	0.40	0.39	0.41	0.24	0.49	0.54	0.56	0.69	0.81	**88**
0.11	0.57	0.02	0.13	0.48	0.24	0.25	0.33	0.36	0.18	0.17	0.28	0.43	**89**
0.60	0.29	0.29	0.40	0.42	0.08	0.69	0.45	0.40	0.46	0.47	0.65	0.73	**90**
0.90	0.85	0.87	0.94	0.63	0.76	0.42	0.28	0.63	0.47	0.97	0.52	0.90	**91**
0.38	0.19	0.77	0.67	0.57	0.54	0.30	0.84	0.88	0.98	1.00	0.83	0.98	**92**
0.89	0.68	0.91	0.96	0.92	0.98	0.56	0.38	0.40	0.75	0.98	0.84	0.98	**93**
0.46	0.15	0.71	0.55	0.74	0.26	0.90	0.31	0.28	0.10	0.62	0.47	0.61	**94**
0.48	0.63	0.67	0.25	0.23	0.52	0.51	0.57	0.27	0.31	0.60	0.48	0.85	**95**
0.47	0.76	0.50	0.20	0.10	0.19	0.09	0.33	0.21	0.28	0.78	0.32	0.55	**96**
0.61	0.59	0.71	0.63	0.47	0.47	0.08	0.13	0.13	0.19	0.88	0.77	0.42	**97**
0.28	0.56	0.99	0.39	0.17	0.72	0.41	0.46	0.39	0.76	0.76	0.33	0.93	**98**
0.48	0.60	1.00	0.79	0.47	0.88	0.47	0.28	0.27	0.84	0.96	0.75	0.97	**99**
0.37	0.43	0.27	0.42	0.32	0.53	0.59	0.64	0.41	0.75	0.50	0.32	0.91	**100**
0.52	0.47	0.76	0.63	0.32	0.61	0.60	0.37	0.63	0.88	0.71	0.27	0.89	**101**

Table of Raup-Crick Similarity index-analysis of bacterial communities across climatic changed (part 3)

95	94	93	92	91	90	89	88	87	86	85	84	83	
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													78
													79
													80
													81
													82
													83
												0.96	**84**
											0.98	0.62	**85**
										0.95	0.97	0.26	**86**
									0.95	0.17	0.83	0.20	**87**
								0.94	0.48	0.56	0.95	0.98	**88**
							0.77	0.95	0.35	0.14	0.14	0.13	**89**
						1.00	0.97	0.64	0.90	0.80	0.68	0.41	**90**
					0.32	0.17	0.55	0.05	0.35	0.32	0.99	0.85	**91**
				0.99	0.59	0.08	0.94	0.28	0.40	0.43	0.46	0.40	**92**
			1.00	1.00	0.29	0.02	0.95	0.26	0.70	0.73	0.99	0.99	**93**
		0.99	0.69	0.63	0.70	0.03	0.49	0.43	0.80	0.83	0.83	0.93	**94**
	0.99	0.89	0.87	0.63	0.90	0.14	0.46	0.21	0.94	0.84	0.56	0.79	**95**
1.00	0.86	0.89	0.89	0.99	0.59	0.43	0.14	0.07	0.43	0.18	0.58	0.69	**96**
1.00	0.95	0.88	0.69	0.79	0.77	0.15	0.27	0.07	0.61	0.37	0.71	0.83	**97**
0.98	0.98	0.99	1.00	0.83	0.52	0.06	0.85	0.63	0.72	0.53	0.34	0.69	**98**
0.99	0.99	1.00	1.00	0.95	0.69	0.12	0.91	0.69	0.81	0.63	0.56	0.81	**99**
0.96	0.88	1.00	0.91	0.96	0.57	0.08	0.36	0.10	0.41	0.70	0.75	0.88	**100**
0.96	0.87	1.00	1.00	0.91	0.47	0.04	0.80	0.27	0.62	0.89	0.61	0.64	**101**

Table of Raup-Crick Similarity index-analysis of bacterial communities across climatic changed (part 4)

	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101
96																																										1.00	0.85	0.97	0.89	0.79
97																																											0.77	0.95	0.72	0.43
98																																												1.00	0.99	1.00
99																																												0.97	1.00
100																																													1.00
101																																													

Raup and Crick similarity index (S_{RC}) with 95% of confidence level indicates the level of similarity among and between bacterial community profiles of each sediment sample. The underlined value implies significant dissimilarity ($S_{RC} < 0.05$), the highlighted value in red indicates significant similarity ($S_{RC} > 0.95$) and the rest indicates similarity occurred by chance ($0.05 < S_{RC} < 0.95$) (Rowan *et al*., 2003; Baxter and Cummings, 2006).

Appendix 12: Tables for basic macro- and micro-morphological characterisation of representative isolates

a) Isolates recovered on brackish sediment sample of SG06-A01

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
1	34	CFE	+	Short fat rod	
2	67	CFE	+	Short fat rod	Like small sausage
3	72, 70	CFE	+	Short thin rod	
4	99	CFE	+	Short thin rod	Some with endospore
5	73,92	FFL	-	Coccus to short round rod	
6	90	CFE	+	Short rod	
7	76, LIGHTER 73	CFU	+	Short thin rod	
8	LIGHTER 70	CCE	+	Short fat rod	
9	90	CFE	+	Short rod	Some with endospore
10	90	CFU	+	Coccus to short round rod	Clumping together
11	90	CRE	+	Short rod	
12	90	CFE	+	Short rod	Slightly curvy
13	90	IFU	+	Short fat rod	
15	73	IFU	+	Coccus to short round rod	
18	93+TRANS	CFE	+	Short fat rod	Some with endospore
19	73	CFE	-	Short thin rod	
21	33	CFE	+	Longer thin rod	
22	70	CRE	+	Longer thin rod	
23	92	CFE	+	Coccus to short round rod	
24	52	CRE	+	Short thin rod	Some with endospore
25	73, SLIMMY TRANS	CFE	+	Coccus to short round rod	
27	DULL, 72, TRANS	CUE	+	Short thin rod	Some with endospore
28	73 SHINNY	CFE	+	Short thin rod	Some with endospore
29	52	RFU	-	Short rod	
30	72	CUE	+	Short fat rod	Like small sausage
32	LIGHTER37, 52	CFL	+	Short rod	
33	70	CFU	+	Short rod	
34	90	CRE	+	Short fat rod	Like small sausage
36	TRANS 105	CFE	+	Short fat rod to coccus	
37	70	CFE	+	Short rod	
39	90	CFE	+	Short rod	
40	52	FRU	+	Coccus to short round rod	
41	LIGHTER 53	IRU	+	Short thin rod	Some with endospore
44	93 TRANS	CFE	+	Tiny thin rod	Some with endospore
45	93 DULL	CFE	-	Short rod	
46	70,73	CUL	+	Very thin rod	Slightly curvy and longer
47	76 SHINNY	CFE	+	Short fat rod	Like small sausage
48	76, TRANS	CRE	-	Thin long rod	
49	SHINE73, DULL TRANS	CUE	-	Tiny coccus	
50	73, 93 SHINNY	CRE	-	Short thin rod	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
	TRANS				
51	73	FFL	-	Short thin rod	
52	74,72	CRE	-	Short rod	
53	LINING71, 70	CRE	+	Short rod	
54	93,73	CRE	+	Short thin rod	
55	LIGHTER72, TRANS 33	CFE	+	Short fat rod	Like small sausage
56	93	CFE	-	Coccus to short round rod	
57	74	CRE	+	Tiny fatter rod	
58	72,70	CFL	+	Short thin rod	Some with endospore
62	LIGHTER72	CFE	+	Very thin short rod	
63	72, 70	CFE	+	Short fat rod	
64	73	IFU	+	Short fatter rod	
65	72,73	CFE	+	Short thin rod	Some with endospore
66	90	CRE	+	Tiny fat rod	
67	LIGHTER 90, DARKER90	CRE	+	Short thin rod	
68	70	CCE	+	Short rod	
70	52	CRE	-	Short fat rod	
71	93 DULL	CFE	+	Short fat rod	
72	LIGHTER 71	CRE	+	Short fat rod	
73	73	CRE	-	Coccus to short round rod	
74	90	CRE	+	Coccus to short round rod	Some rods/some coccus/some with spores
74	90	CRE	+	Short thin rod	Some with endospore
75	70	FFU	+	Short fat rod	Like small sausage
76	52	CFE	+	Short thin rod	
77	71,70	CFE	+	Short fatter rod	
78	52	CRE	+	Short thin rod	
79	53	IFU	+	Short fatter rod	Some with endospore
80	TRANS73	CFU	+	Short thin rod	
82	70	CRE	+	Short fatter rod	Like small sausage
84	73, 93 TRANS	CFE	-	Short thin rod	
86	52	CCRE	-	Short fatter rod	
87	70	CRE	-	Short fatter rod	Like small sausage
88	73	CRE	+	Rod	
89	70	CFE	+	Short thin rod	Some with endospore
91	73, SLIMMY WHITE/TRANS	CFE	-	Coccus to short round rod	
92	99	CFE	+	Short fat rod	Some with endospore
93	93	CFE	+	Very thin short rod	Some with endospore
95	52	CFE	+	Very thin short rod	Some with endospore
96	52	CFU	+	Short fatter rod	
98	LIGHTER 70	CFE	+	Short fat rod	Some with endospore
99	LIGHT 70, TRANS	CRE	-	Short rod	
100	102 TRANS	CFE	-	Very thin, longer straight rod	
101	73+TRANS	CRE	-	Short thin rod	
102	73	CFE	+	Very thin short rod	Some with endospore
103	70, 70+TRANS	CUE	-	Coccus to short round rod	
104	93+TRANS	CFE	+	Short fat rod	
107	73	FFU	-	Coccus to short round	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
				rod	
108	73	RFU	-	Short fatter rod	
111	57	CFE	-	Thin rod	
112	92	CFE	-	Coccus to short round rod	
114	70+TRANS	CFE	+	Coccus to short round rod	
115	52	IFU	+	Coccus to short round rod	
116	52	CRE	+	Short thin rod	Some with endospore
117	70	CFE	-	Short fat rod	
118	39, 52	CFU	-	Short thin rod	
120	53,52	CFE	-	Coccus to short round rod	
121	71, 70	CFE	+	Shorter rod	
124	52	CCE	+	Short thin rod	
127	92	CRE	+	Coccus	
128	71,70	CFE	-	Short rod	
129	73	CCE	+	Short fatter rod	
131	83	CRE	-	Coccus	
132	98	CCE	+	Diploid coccus	
133	67	CCE	-	Diploid coccus	
134	70	CFU	+	Short fatter rod	
136	52	CFE	+	Short thin rod	
137	71,70	CUE	+	Short round rod	
138	93	CFE	+	Diploid coccus	
139	70	CRE	-	Short thin rod	
140	73	CRE	-	Tiny short rod to coccus	
141	73	CRE	-	Tiny coccus	
142	73	CFE	+	Coccus	Some diploid
144	33	FFU	+	Short thin rod	
146	82	FFU	-	Short fat rod	
147	91,54,90	CCE	+	Short thin rod	
148	31	CRU	-	Short thin rod	
149	31	CRU	-	Short thin rod	Shinny
150	TRANS 72	CFE	-	Short thin rod	
152	TRANS 87	CRE	-	Short thin rod	
153	53,52	CRE	-	Short thin rod	
154	32	CFE	+	Short thin rod	Some with endospore
155	33	CFL	-	Coccus to short round rod	
157	87, TRANS	CFE	-	Short thin rod	
159			-	Short fat rod	
164	70	CFE	-	Short thin rod	
165			+	Short fat rod	Some with endospore
168	33	CFE	+	Short thin rod	
170	70, 33	CFE	+	Short thin rod	
171	33	CFE	-	Short thin rod	
172	89	CFL	-	Short thin rod	
174	76	CFE	+	Short thin rod	
176	LIGHTER 72, 72	CFL	+	Short thin rod	
177	70	CFE	+	Thin rod	Some with endospore
178	70 STICKY	CFE	+	Thin rod	Some with endospore
179	102 TRANS	CFE	-	Short thin rod	
182	70 DULL	FFL	-	Thin rod	
185	TRANS87	CFE	+	Short thin rod	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
187	MILKY TRANS	CFE	+	Short thin rod	Some with endospore
188	TRANS	CFE	-	Short thin rod	
190	TRANS, 76	CFE	-	Short thin rod	
191	76	CFE	+	Short thin rod	Some with endospore
194	72,TRANS	CFE	+	Short rod	Some with endospore
195	53,52	IRU	-	Short thin rod	
197	32,70	CFE	+	Short fat rod	
199	54,72	CFE	-	Coccus to short round rod	Diploid
200	LIGHTER72, SHINNY	CFE	-	Short thin rod	
202	90	CFE	-	Short thin rod	
204	SHINNY 33	CFE	+	Short thin rod to coccus	Some with endospore
205			-	Short rod	
206	LIGHTER76	CFE	-	Short rod	Dull
207	70	RFL	+	Staphylococcus	
208	33,32	CRL	+	Long thin rod/some Short rod	
211	33,LINING31	FFU	-	Short thin rod	
215	87 TRANS	CFE	+	Tiny coccus	
216	70	FFU	-	Rod	
217	70, TRANS	FFE	+	Staphylococcus	
219	HOLE, 33	CFE	-	Short rod	
220	SHINNY70	FFU	-	Very thin short rod	
221	73	FFE	+	Short thin rod	
223	33	CFE	-	Short thin rod	
224	LIGHTER72	CFE	+	Coccus to short round rod	
226	33	IFL	+	Short thin rod	
227	70SLIMMY, 73	FRU	-	Short rod	
228	73	FFU	-	Short rod	
229	72	CRE	+	Staphylococcus	
231	70	CRL	-	Coccus to short round rod	
232	87	CRE	+	Small coccus	
233	57,76	CFE	-	Short thin straight rod	
236	49	FFU	+	Short thin straight rod	
238	71,72	CRE	+	Tiny coccus	
239	71	RFU	+	Short thin rod	
240	70, TRANS	CRE	-	Short fat rod	
241	SLIMMY70, 73	FRU	-	Short thin rod	
242	33,76	IFL	+	Short thin rod	
244	LIGHTER 37	IFU	+	Short rod	
247	71,70	CFE	+	Straight thin rod	
248	LIGHTER 72	CFE	-	Short thin rod in a chain	Chained like
249	72, SLIMMY TRANS	CFE	-	Short thin rod	
250	38,39	CRE	+	Straight thin rod	
251	LIGHTER 53	CCE	-	Curvy thin rod	
252	73	RRU	-	Short thin rod	
254	LIGHTER 74,76	IUU	+	Coccus to short round rod	
255	TRANS	CFE	-	Coccus	
256	LIGHTER 76	IFU	+	Coccus to short round rod	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
257	TRANS 72	CRE	+	Coccus	Slimmy
259	LIGHT 53, TRANS	CRE	-	Short thin rod	
261	70 DULL	CFE	+	Straight thin rod	
264	73	FFU	-	Straight thin rod	
265	70	RRU	+	Straight thin rod	
266	70	FFU	-	Short thin rod	
267	53, 70TRANS	IUU	-	Short thin rod	
268	70	RFU	-	Short thin rod	
269	73	RRU	-	Straight thin rod	
270	LIGHT70	FFU	-	Straight thin rod	
271	76	CFE	-	Thin rod	
273	52	FFU	-	Straight thin rod	
275	70	FRE	+	Short fatter rod	
276	70	FRU	+	Straight thin rod	Embed
277	70	FFL	-	Short fat rod	Embed
278	70	FRU	-	Straight thin rod	Embed
279	91,90	CFU	+	Short thin rod	Some with endospore
281	33	FFE	-	Thin rod	
282	70	FFU	+	Staphylococcus	
283	73	IFU	+	Straight thin rod	
285	90	CFU	+	Tiny coccus	
287	90	CFE	-	Tiny small rod	
288	34,53	IRU	+	Staphylococcus	
289	52	FRU	+	Short fatter rod	
290	72LIGHT, TRANS	FFU	+	Thin rod	
291	LIGHTER52	FFL	+	Straight short rod	
292	54	CFE	+	Thin long rod	
294	87, TRANS	CFE	+	Clumping coccus	
295	90 TRANS	CFE	+	Big oval/coccus	
296	76	IFU	+	Tiny coccus	
297	TRANS-DULL	CFE	+	Staphylococcus	
300	32,33+TRANS,70	CFE	+	Straight thin rod	
301	72	RFU	+	Short fat/rounder rod	
302	84	FFU	-	Short rod	
304	TRANS	CFE	+	Staphylococcus	
305	52	FRU	+	Straight thin rod	Embed
306	52	FRU	+	Thin rod	Embed, with terminal endospore
308	70	FRL	+	Short rod	
310	92	CFE	+	Short rod	
311	TRANS	CFE	-	Thin rod; some longer rod	
312	70,33	FRE	+	Short fat rod	
313	52	FFU	+	Straight thin rod	
314	33, L33	RRU	+	Staphylococcus	
315	33, 73, 33	RFC	+	Straight thin rod	
318	33	IFU	-	Straight thin rod	
320	102, TRANS	CFE	-	Tiny fat rod	
321	88, LIGHT87	CUE	-	Short rod	
327	52	FRU	+	Very long rod	
329	52+TRANS	IFE	+	Straight rod	
332	73	FCL	+	Straight thin rod	
334	73	RFE	+	Short fat rod	
335	72	CUE	+	Tiny coccus	
336	52	FRU	-	Short single thin rod	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
338	90,73	CFU	+	Single fatter rod	
340	48	CCE	+	Small coccus	
342	90	CFE	-	Straight short thin rod	
343	LIGHTER 90	CFE	+	Straight short thin rod	Some with endospore
345	70	CFE	+	Short thin rod	
346	71	CFE	-	Single thin rod	
347	53	CFE	+	Single thin rod	
348	72	CUE	-	Short thin rod	
349	54+ transp	CFE	+	Single rod	
350	71,70	CFUE	-	Single long straight rod	
351	67	IFU	+	Single short straight rod	
352	71	FFL	-	Short fatter rod	
353	71,70	CFE	+	Long thin straight rod	
354	LIGHTER72	FRL	+	Tiny coccus in chain/staphylococcus	
357	33, TRANS93, 33	CFE	+	Diplococoid/coccus in pair	
358	52	FFU	-	Straight rod	
359	70	CRE	+	coccus	Some diploid
360	TRANS 105	CFE	+	Short thin rod	
362	76	CFE	+	Short thin rod	
363	72 SHINNY	CRE	-	Tiny thin short rod	

There was a total number of 256 taxa were detected to form on both medium. Isolate no. 1 to 41 and 96-113 were formed on MA medium while isolate no. 42-95 were formed on TPA medium. Colour codes were referred to NBS Colour Name Charts (Kelly, 1958; NBS, 1964). Shapes were determined by their forms, elevations and margins. **Form**: Circular (C), Irregular (I) Filamentous (F) and Rhizoid (R); **Elevation**: Raised (R), Convex (C), Flat (F), Umbonate (U) and Crateriform (Cr); **Margin**: Entire (E), Undulate (U), Filiform (F), Curled (C) and Lobate (L). For example, CRE represents Form C= circular, Elevation R= raised and Margin E= entire, respectively in this sequence. Gram – isolates were stained red/ pink while Gram + were stained purple/ violet. Colour code i.e. 71.70; 71 represents the inner colour of the colony while the latter number 70 indicates the colour of the outer layer of the colony.

b) Isolates detected from freshwater sediment sample of B (N) 05

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
1	32	CRE	-	Rod	
2	33	CRE	+	Short rod	
3	92	CRE	-	Short rod	
5	121	CF+UE	-	Short rod	
7	31	CRE	-	Coccus	
8	70.73	CRE	+	Coccus	
9	32	IFE	-	Rod	
10	51	IFE	+	Rod	
11	66.49	CF+UE	-	Coccus	
12	33.9	CRE	+	Short rod	
13	67	CUE	-	Tiny coccus	
15	104	CCE	+	Coccus	
16	89	CCE	-	Concave rod + short rod	
17	70.83	CUE	-	Short rod	
19	85	CUE	-	Short fat rod	
21*	52+ TRANS	FCU	-	Long rod	
23	89	CUE	+	Short rod-> coccus	
24	89	CRE	-	Short rod	
25	73	CCE	+	Short rod-> coccus	
26	48	CCE	+	Short rod-> coccus	
27	35	CCE	-	Short rod-> coccus	
29	35	CCE	+	Thin rod	Some have endospores
30	34	ICU	+	Coccus	
31*	70.73	FCU	-	Coccus	
34*	93.92	FUU	+	Very long thin rod	
35*	72	CFE	+	Coccus-> short rod	
37	32.33	CRE	+	Short rod	
39	66	CRE	+	Coccus	
40	71	CCE	+	Coccus	
41	92	CCE	+	Tiny coccus	
43	97	CRE	+	Coccus-> short rod	
45	84	CCE	+	Short rod	Some have endospores
46	105	CF+UE	-	Coccus-> short rod	
47*	66	FCU	-	Coccus	
48*	73	FCU	-	Thin rod	Longer rod
49	33	IFE	-	thin rod	
50	32.90	CFE	-	Short fat rod	Some concave/vibrio
51	9	CFE	-	Short fat rod	
52	37.71.70	CF+UE	-	Short fat rod	
53	32.33	CUE	+	Short rod	
55*	32	FRC	+	Thin rod	
56	33	CFE	-	Short fat rod	
58	35	CUE	-	Coccus-> short rod	
59	92	CUE	+	Coccus-> short rod	
60	48	CRE	-	Short fat rod	
62	73	CFE	+	Short fat rod	
63	49	CRE	+	Short fat rod	
64	67	CRE	+	Coccus	
66	89.73	CUE	+	Short fat rod	
68	84	CRE	+	Short fat rod	Shiny
70	35	CUE	+	Coccus	
72	50.70	CUE	+	Short fat rod	
74	71.83	CUE	-	Short fat rod	
75	92	CF+UE	-	Coccus-> short rod	
77	50.49	CUE	+	Coccus-> short rod	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
79	83	CRE	+	Short fat rod	
80	70.92	CFE	-	Coccus-> short rod	
81	92	ICU	-	Short fat rod	
82	50	CCE	+	Short fat rod	
83	40	CRU	+	Short thin rod	
84	105	CFE	-	Coccus-> short rod	
85	48.34	CRE	+	Thin long rod	Some long rod, some shorter rod
87	50.53	CUE	+	Short fat rod	
89	50	CR+UE	+	Short fat rod	
91	52.73	CCE	+	Rod	Fat short rod
92	53.35	CCE	-	Coccus	
93	82	CCE	+	Short fat rod	
95	82	CCE	+	Coccus	
96	48.53	CUE	+	Tiny coccus	
97	82	CRE	+	Coccus	
99	86	CCE	-	Coccus	
100	89	CRE	+	Coccus	
101	66.49	CRE	+	Coccus	Clumping
103	52	CCE	+	Coccus-> short rod	
105	50	CC+UE	+	Coccus-> short rod	
106	52	CUE	-	Coccus-> short rod	
107	73	CCE	+	Coccus	
108	71.67	CRE	-	Coccus-> short rod	
109	85.67	CRE	-	Tiny coccus	
112	31	CRE	+	Short fat rod	
113	89.92	CFE	-	Tiny coccus	
114	35.98	CRE	-	Tiny coccus	
116	49.86	CUE	+	Tiny coccus	
118	35	CRE	+	Tiny coccus	
120	31	CCE	+	Tiny coccus	
122	34	CRE	-	Short thin rod	
123	48.53	CUE	+	Tiny coccus	
124	85	CRE	+	Tiny coccus	
125	34	CCE	-	Short thin rod	
126			+	Short fat rod	
128	33	CRE	+	Coccus	
130	52	CCE	-	Coccus-> short rod	
131	31	CRE	+	Coccus-> short rod	
132	82.83	CRE	+	Tiny coccus	
133	52	CCE	+	Tiny coccus	
134	71	CUE	+	Short rod	Bit pointy rod
136*	51+TRANS	FFU	+	Tiny short rod	
138	97	CRE	+	Tiny coccus	
139	53.52	CUE	+	Tiny coccus	
140*	69	FRL	+	Short rod	
142*	69	CRE	-	Tiny coccus-> short fat rod	
143	49	CUE	+	Tiny coccus-> short fat rod	
144	35	CRE	+	Coccus-> short fat rod	
146	82	CCE	+	Tiny coccus	
147	66	CUE	+	Tiny coccus	Short rod
148	83	CCE	+	Tiny coccus	
149	33	CFE	+	Short rod	Bit pointy rod
150	33	CFE	+	Tiny short rod	Bit pointy rod

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
151	90.33	CFE	+	Tiny short rod	Bit pointy rod
153	82	CFE	+	Tiny coccus-> short fat rod	
155	71	RUU	-	Tiny short rod	
157	92	CRE	+	Tiny coccus-> short fat rod	
160*	50	FCU	+	Coccus-> short rod	
162	67.86	CFE	+	Coccus	
163	48	CRE	+	Coccus	
164	39.35	CFE	+	Short thin rod	
165	48.49	CRE	+	Coccus	
167	50	CCE	+	Coccus-> short fat rod	
169	48.49	CRE	+	Tiny coccus	
170	48	CRE	+	Short fat rod	
171	70	CCE	+	Tiny coccus-> short fat rod	
173	83	CCE	+	Tiny coccus-> short fat rod	
175	99.98	CRE	+	Tiny coccus	
177	97.98	CF+UE	+	Tiny coccus	
179			-	Tiny coccus	Super tiny
180	71	RRU-Shiny	+	Tiny coccus	Some have endospores
181	34	CFE	+	Short thin rod	
182	72	CFE	+	Coccus-> short fat rod	
184	97	CFE	+	Coccus	
185	53.83	CUE	+	Tiny coccus	
186	73	CRE	+	Tiny coccus	
187	73	CRE	-	Tiny coccus-> short fat rod	
188	50	CUE	+	Coccus-> short fat rod	Bit pointy rod
190	71	CCE	-	Tiny coccus	
191	71	CCE	-	Tiny coccus	
193	70	CCE	-	Tiny coccus	
195	50	CCE	+	Tiny coccus	
199*	33	ICE	+	Thin rod	Sporulate
200*	70.33	IRU	+	Tiny coccus	
201	54	CFE	-	Coccus-> short fat rod	
203	50	CCE	-	Coccus	
207	49-WATERY	CRE	+	Coccus	
211	34	CCE	-	Tiny coccus	
212	83	CUE	+	Tiny coccus	
214	33	CRE	+	Coccus	Concave rods
221	49	CCE	-	Coccus	
223	71	CCE	-	Coccus	
225	89.92	CRE	-	Coccus	
228	71	CRE	-	Coccus	
230	86	CCE	-	Coccus	
231	35	CCE	-	Coccus	
233	92	CRE	-	Coccus	
234	99	CCE	-	Coccus	
235	73	CCE	-	Coccus	
236	35 DULL	CCE	-	Coccus	

Isolate No.	Colour Code	Form, Elevation, Margin	Gram +/-	Shape	Comments
238	82.83	CCE	-	Coccus	
240	49 LIGHTER	CCE	-	Coccus	
242	83 DULL	CCE	-		
244	70	CCE	+	Coccus	
246	67	CCE	-	Coccus	
247*	32	FFC	-	Short rod	Some fat, some thin
248	69	CRE	-	Thin rod	

There was a total number of 162 taxa were detected to form on both medium. Isolate no. 1 to 148, 188-248 and were formed on TPA medium while isolate no. 149-187 were formed on MA medium. Isolate numbers indicated with * were tested for acid-fast staining test and all were non-acid fast.

c) Isolates recovered from freshwater sediment sample of B (N) 07

Isolate No.	Colour Code	Form, Margin, Elevation	Gram +/-	Shape	Comments
1	102	CRE	+	Tiny coccus	
4	33	CFE	+	Tiny coccus	
5	53	CRE	+	Coccus	
7	52	CRE	-	Super tiny coccus	
8	53	CCE	-	Tiny coccus	
9	92	CCE	+	Tiny coccus	
10	92	CRE	+	Coccus	
11	97	CRE	-	Tiny coccus	
12	68.70	CRE	-	Tiny coccus	
13	89	CCE	-	Tiny coccus	
15	92	CCE	+	Tiny coccus-> short rod	
16	92	CRE	+	Tiny coccus	
18	101	CCE	+	Coccus	
20	53	CRE	+	Coccus	
23	73 SHINNY	CRE	+	Coccus-> short rod	
26	98	CCE	-	Coccus	
27	97	CRE	+	Coccus	
29	70	CRE	-	Coccus	
30	83	CCE	-	Coccus	
31	73	CRE	-	Coccus	
32	67.83	CUE	+	Tiny coccus	
33	68.67	CRE	+	Tiny coccus	
34	87	CRE	+	Tiny coccus	
36	73	CFE	+	Coccus	
37	34.50	CFE	+	Coccus-> short rod	
38	72.70	CFE	+	Short thin rod	Some long thin rod+ some with endosprores
39	98	CCE	-	Coccus	
40	49	CCE	-	Coccus	
41	73	CCE	-	Tiny coccus	
42	72	CRE	-	Tiny coccus	
44	87	CRE	+	Tiny coccus	
47	83	CRE	+	Coccus	
48	92	CRE	-	Tiny coccus	
51	89.92	CUE	-	Coccus	
54	66	CCE	+	Dicoccus	
55	90	CFE	-	Coccus-> short fat rod	
56	32	CUE	-	Coccus-> short rod	
59	53	CCE	+	Short rod	Concave
62	70	CRE	+	Tiny coccus	
63	31	CCE	+	Coccus-> short rod	
64	67	CCE	-	Tiny coccus	
65	72	CRE	+	Coccus-> short rod	
67	39	CRE	+	Coccus-> short rod	Some with endosprores
68	67.49	CCE	-	Coccus	
71	35	CCE	-	Coccus	
74	86	CCE	-	Coccus	
76	89.99	CRE	-	Coccus	
79	28	IFU	-	Short rod	
80	49	CCE	-	Coccus	
81	9	CCE	+	Thin rod	Short + moderate
82	70.92	CUE	-	Coccus	
85	37.72	FFU	-	Coccus	
88	96.76	CCE	-	Coccus	
90	32.33	CRE	+	Coccus	

Isolate No.	Colour Code	Form, Margin, Elevation	Gram +/-	Shape	Comments
92	73	CCE	+	Short rod	
93	70	ICU	-	Coccus	
94	71	CCE	-	Coccus	
95	92	CCE	-	Short thin rod	
96	73	CRE	-	Coccus	
99	70	CCE	+	Coccus	
100	52	CCE	+	Coccus	
102	97	CCE	-	Coccus	
103	39.33	CFE	-	Thin rod	Concave, some short, some moderate
105	92	CFE	-	Coccus	
106	53	CCE	+	Coccus	
107	53.73	CRE	-	Coccus	
109	85	CFE	+	Coccus	
112	86	CRE	+	Coccus	
113	89	CCE	-	Coccus	

There was a total number of 69 taxa were detected to form on both medium. Isolate no. 1 to 41 and 96-113 were formed on MA medium while isolate no. 42-95 were formed on TPA medium.

Appendix 13: Tables of nucleotide differences

***Dermacoccus* test strains**

	B. humi	*B. hedensis*	*C. indicus*	*D. terragena*	*D. abyssi*	*D. barathri*	*D. nishinomiyaensis*	*D. profundi*	F124T	F142T	F156T	F218T	F195T	*Fl. alba*	*K. sedentarius*	*L. mongoliensis*	*R. suwonensis*	*T. marinus*
B. hedensis	65																	
C. indicus	73	64																
D. terragena	44	84	80															
D. abyssi	74	79	54	85														
D. barathri	72	77	52	85	2													
D. nishinomiyaensis	79	79	61	90	21	20												
D. profundi	73	78	53	85	3	1	20											
F124T	75	75	59	89	18	17	3	18										
F142T	69	69	56	81	17	17	9	18	6									
F156T	73	73	57	86	15	15	7	16	4	2								
F218T	85	86	65	99	28	27	13	28	8	13	12							
F195T	70	69	59	80	18	17	4	18	3	5	3	12						
Fl. alba	94	80	73	100	81	81	79	82	77	77	74	87	79					
K. sedentarius	101	131	85	102	77	77	80	78	76	74	75	91	75	105				
L. mongoliensis	92	91	77	88	52	52	54	53	54	56	50	75	61	75	105			
R. suwonensis	80	69	65	90	72	71	65	71	61	61	63	68	59	56	93	71		
T. marinus	14	66	74	46	77	75	81	76	78	71	76	87	70	92	102	81	81	
Y. lutea	82	87	34	88	61	59	63	59	60	59	58	70	60	71	104	72	64	81

***Dietzia* test strains**

	A. subflavus	*C. diphtheriae*	F130T	*D. aerolata*	*D. alimentaria*	*D. aurantiaca*	*D. cercidiphylli*	*D. cinnamea*	*D. kunjamensis*	*D. lutea*	*D. maris*	*D. natronolimnaea*	*D. papillomatosis*	*D. psychralcaliphila*	*D. schimae*	*D. timorensis*	*G. bronchialis*	*H. altamirensis*	*M. brevis*	*M. tuberculosis*	*N. asteroides*	*R. rhodochrous*	*S. rotundus*	*S. piniformis*	*S. niigatensis*	*T. biformata*	*T. paurometabola*	*T. otitidis*
C. diphtheriae	143																											
F130T	108	11																										
D. aerolata	111	11	21																									
D. alimentaria	122	13	25	26																								
D. aurantiaca	112	10	25	10	24																							
D. cercidiphylli	120	12	4	24	35	28																						
D. cinnamea	113	11	31	26	47	30	37																					
D. kunjamensis	120	12	31	30	29	32	37	36																				
D. lutea	108	11	19	19	24	19	19	21	29																			
D. maris	111	11	23	19	28	21	25	26	13	18																		
D. natronolimnaea	123	12	5	26	38	31	11	38	39	26	30																	
D. papillomatosis	131	13	50	46	56	48	58	24	60	43	50	60																
D. psychralcaliphila	132	14	16	35	47	39	30	58	49	40	45	29	73															
D. schimae	112	11	24	18	29	20	26	30	14	19	3	31	51	46														
D. timorensis	111	11	46	38	60	46	50	42	56	39	45	54	65	68	46													
G. bronchialis	98	12	10	97	11	97	10	99	11	96	10	11	11	12	10	94												
H. altamirensis	3	12	10	10	10	10	10	10	10	99	10	10	11	11	10	98	87											
M. brevis	94	11	96	88	10	89	10	94	98	92	87	10	11	11	93	90	62	82										
M. tuberculosis	81	12	11	11	13	11	12	11	12	11	10	12	13	14	11	11	10	73	89									
N. asteroides	99	13	11	10	11	10	11	93	10	10	99	11	11	13	10	10	92	88	71	96								
R. rhodochrous	93	12	82	79	94	83	85	76	87	77	77	89	98	10	78	75	80	82	66	10	82							
S. rotundus	136	14	10	10	12	10	11	11	11	11	10	12	13	13	10	12	12	11	10	13	10	95						
S. piniformis	102	12	10	10	11	10	10	94	11	10	10	11	11	12	10	98	65	91	63	10	89	76	11					
S. niigatensis	108	12	10	10	11	10	11	97	11	10	10	11	11	12	10	10	90	98	92	11	79	76	10	80				
T. biformata	105	11	97	94	10	96	10	98	10	96	93	10	11	12	94	95	90	91	76	10	79	72	11	88	92			
T. paurometabola	102	11	77	75	86	75	81	69	79	74	68	86	91	98	69	74	99	90	84	10	88	79	10	10	10	86		
T. otitidis	151	13	13	13	14	14	14	14	15	14	14	14	16	15	14	14	14	13	15	15	15	15	17	15	15	16	15	
W. muralis	104	13	11	11	13	11	12	10	12	11	11	12	12	14	11	11	69	92	73	10	76	73	11	86	87	10	93	15

***Leifsonia* test strains**

	A. jenensis	F96T	*L. antarctica*	*L. aquatica*	*L. bigeumensis*	*L. cynodontis*	*L. kafniensis*	*L. kribbensis*	*L. lichenia*	*L. naganoensis*	*L. pindariensis*	*L. poae*	*L. psychrotolerans*	*L. rubra*	*L. shinshuensis*	*L. soli*	*L. xyli subsp. cynodontis*	*M. lacticum*	*M. viridarii*	*O. fritillariae*	*P. flavus*
F96T	74																				
L. antarctica	60	64																			
L. aquatica	86	27	77																		
L. bigeumensis	83	68	57	68																	
L. cynodontis	82	20	65	24	56																
L. kafniensis	48	62	36	70	56	60															
L. kribbensis	61	79	71	63	71	64	52														
L. lichenia	70	10	55	29	47	23	50	63													
L. naganoensis	76	23	66	11	59	16	61	56	21												
L. pindariensis	69	68	43	74	60	61	38	69	57	70											
L. poae	66	24	53	34	59	32	47	79	13	28	49										
L. psychrotolerans	54	53	29	60	45	52	13	49	45	52	28	41									
L. rubra	86	77	59	76	62	76	53	76	69	66	71	65	48								
L. shinshuensis	77	10	67	30	57	13	64	68	15	23	71	23	56	79							
L. soli	69	2	53	21	40	19	48	55	6	17	52	13	47	62	7						
L. xyli subsp. cynodontis	82	20	65	24	56	0	60	64	23	16	61	32	52	76	13	19					
M. lacticum	72	77	80	80	88	76	61	69	76	69	80	71	53	89	79	72	76				
M. viridarii	61	59	32	69	60	60	29	70	56	61	2	49	26	60	62	52	60	68			
O. fritillariae	60	68	62	77	79	75	47	72	60	66	66	54	43	75	72	58	75	57	54		
P. flavus	80	72	63	76	88	72	55	73	64	66	72	68	46	71	74	59	72	66	60	38	
R. vestalii	91	83	65	80	63	82	59	77	75	73	70	71	55	8	86	67	82	96	66	79	75

***Rhodococcus* test strains** (part 1)

	C. diphtheriae	*G. bronchialis*	*M. brevis*	*N. asteroides*	F42M	F152M	*R. aetherivorans*	*R. artemisiae*	*R. baikonurensis*	*R. canchipurensis*	*R. cerastrii*	*R. cercidiphylli*	*R. coprophilus*	*R. corynebacterioides*	*R. defluvii*	*R. erythropolis*	*R. fascians*	*R. globerulus*	*R. gordoniae*	*R. hoagii*	*R. imtechensis*	*R. jialingiae*
G. bronchialis	129																					
M. brevis	121	64																				
N. asteroides	133	93	75																			
F42M	154	117	107	99																		
F152M	134	102	82	75	57																	
R. aetherivorans	119	77	65	78	82	77																
R. artemisiae	132	89	72	87	79	81	43															
R. baikonurensis	123	90	71	63	42	21	62	67														
R. canchipurensis	126	74	68	69	84	59	50	58	46													
R. cerastrii	123	87	73	70	66	56	67	79	42	52												
R. cercidiphylli	130	95	83	75	86	63	68	85	49	58	7											
R. coprophilus	122	98	70	72	62	67	46	39	53	53	65	67										
R. corynebacterioides	128	83	60	67	77	64	48	64	49	45	50	59	58									
R. defluvii	115	80	65	50	72	66	49	58	50	28	53	54	56	43								
R. erythropolis	124	95	75	58	54	26	57	64	11	41	42	46	47	43	48							
R. fascians	122	98	73	69	73	60	66	81	46	55	11	11	61	51	54	43						
R. globerulus	126	94	78	70	66	32	68	76	15	52	41	50	62	51	49	23	48					
R. gordoniae	115	81	68	74	76	72	43	29	58	54	69	70	43	55	55	55	72	67				
R. hoagii	129	83	71	59	85	67	56	72	51	42	52	62	64	48	19	57	61	54	56			
R. imtechensis	123	105	91	74	91	58	73	84	44	52	52	68	60	66	33	39	51	40	62	42		
R. jialingiae	138	103	88	75	63	18	74	78	3	57	39	61	60	60	49	18	46	23	70	62	55	
R. jostii	130	101	79	73	76	54	63	78	39	47	48	53	56	52	43	35	47	29	67	53	33	50

***Rhodococcus* test strains** (part 2)

	C. diphtheriae	*G. bronchialis*	*M. brevis*	*N. asteroides*	F42M	F152M	*R. aetherivorans*	*R. artemisiae*	*R. baikonurensis*	*R. canchipurensis*	*R. cerastrii*	*R. cercidiphylli*	*R. coprophilus*	*R. corynebacterioides*	*R. defluvii*	*R. erythropolis*	*R. fascians*	*R. globerulus*	*R. gordoniae*	*R. hoagii*	*R. imtechensis*	*R. jialingiae*
R. koreensis	112	88	67	63	72	53	57	62	38	32	42	46	53	48	23	35	40	34	63	39	16	49
R. kroppenstedtii	176	124	113	121	124	94	80	113	77	95	55	98	114	58	55	99	80	107	92	96	111	106
R. kunmingensis	119	93	81	45	84	65	57	75	49	51	46	53	67	49	32	51	54	48	60	30	47	48
R. kyotonensis	125	89	82	72	77	63	65	82	48	64	25	31	66	64	62	47	30	52	64	65	57	45
R. maanshanensis	124	99	73	66	68	55	60	72	40	49	49	50	46	57	49	31	45	42	62	57	38	38
R. marinonascens	118	91	71	60	68	48	56	66	33	43	50	54	43	51	38	28	48	34	52	48	32	40
R. nanhaiensis	120	91	70	64	71	54	49	60	39	43	55	62	54	54	42	41	61	44	44	46	36	37
R. opacus	117	91	69	56	80	58	57	67	43	35	51	56	53	47	28	38	50	37	55	37	16	53
R. percolatus	123	92	70	60	77	53	63	72	40	41	49	55	53	53	35	36	48	35	61	45	26	52
R. phenolicus	115	82	62	75	64	69	40	35	54	32	59	65	34	43	35	53	61	59	41	49	52	56
R. pyridinovorans	131	93	71	84	77	83	38	23	69	63	78	82	36	60	65	62	76	78	13	69	77	80
R. qingshengii	130	95	80	67	55	18	66	72	3	51	39	53	56	52	49	14	46	19	62	58	47	3
R. rhodnii	114	79	62	75	91	72	39	64	57	45	61	66	59	47	57	51	60	59	49	60	62	65
R. rhodochrous	129	88	71	82	66	73	41	33	59	56	67	77	35	54	56	58	72	68	21	57	67	61
R. ruber	129	92	72	82	79	82	7	42	67	54	76	79	41	50	55	63	74	74	40	64	69	75
R. triatomae	124	80	59	61	77	55	44	60	40	32	45	55	56	28	34	35	45	44	45	42	56	51
R. trifolii	122	76	54	64	72	67	47	62	53	38	42	40	56	17	39	48	39	56	57	45	58	52
R. tukisamuensis	127	96	77	75	71	60	60	79	49	44	53	58	58	55	46	41	56	42	63	51	44	46
R. wratislaviensis	112	89	67	52	77	55	54	64	40	32	48	53	50	44	23	34	47	36	52	32	10	51
R. yunnanensis	120	89	77	67	80	58	68	80	44	54	12	16	67	52	51	42	16	44	70	56	49	53
R. zopfii	122	86	68	78	65	72	33	27	57	36	70	74	29	49	40	54	68	63	42	55	56	68
S. niigatensis	128	93	93	81	100	97	73	80	83	70	85	93	90	77	52	82	91	81	71	65	79	84
W. muralis	140	69	75	77	94	99	81	82	84	82	87	92	90	78	65	89	88	90	79	66	85	96

***Rhodococcus* test strains** (part 3)

	R. jostii	*R. koreensis*	*R. kroppenstedtii*	*R. kunmingensis*	*R. kyotonensis*	*R. maanshanensis*	*R. marinonascens*	*R. nanhaiensis*	*R. opacus*	*R. percolatus*	*R. phenolicus*	*R. pyridinovorans*	*R. qingshengii*	*R. rhodnii*	*R. rhodochrous*	*R. ruber*	*R. triatomae*	*R. trifolii*	*R. tukisamuensis*	*R. wratislaviensis*	*R. yunnanensis*	*R. zopfii*	*S. niigatensis*
R. koreensis	28																						
R. kroppenstedtii	107	103																					
R. kunmingensis	55	44	77																				
R. kyotonensis	54	54	89	56																			
R. maanshanensis	30	35	87	60	51																		
R. marinonascens	26	30	104	52	53	28																	
R. nanhaiensis	44	35	74	50	61	37	29																
R. opacus	24	18	104	40	56	33	21	29															
R. percolatus	22	22	109	48	54	31	23	36	11														
R. phenolicus	55	41	57	58	65	57	50	53	45	51													
R. pyridinovorans	78	71	116	70	76	67	67	60	70	74	42												
R. qingshengii	42	41	100	48	47	38	37	37	46	44	56	72											
R. rhodnii	57	57	103	52	59	56	54	57	53	57	47	56	61										
R. rhodochrous	70	63	106	60	65	66	59	55	60	64	35	15	61	51									
R. ruber	70	64	107	64	72	65	63	54	62	67	35	34	71	48	37								
R. triatomae	44	37	81	34	56	49	44	43	36	43	37	52	43	30	48	50							
R. trifolii	55	49	24	51	59	60	51	60	51	56	42	58	52	49	55	51	30						
R. tukisamuensis	35	41	82	53	60	29	35	47	35	38	52	72	46	58	67	67	46	56					
R. wratislaviensis	26	12	101	37	53	34	22	26	6	14	42	67	43	52	57	59	33	48	35				
R. yunnanensis	45	43	98	48	20	48	48	57	48	47	66	79	47	61	68	77	48	44	54	45			
R. zopfii	59	42	104	65	66	58	50	53	49	54	17	38	60	47	32	30	44	51	61	46	71		
S. niigatensis	81	72	117	65	93	84	73	80	72	78	71	79	85	77	75	83	66	73	76	70	86	75	
W. muralis	89	76	128	76	91	96	86	86	80	82	73	87	92	86	80	82	76	77	96	75	86	74	90

Appendix 14: Tables of percentage similarities (%)

***Dermacoccus* test strains**

	B. humi	*B. hedensis*	*C. indicus*	*D. terragena*	*D. abyssi*	*D. barathri*	*D. nishinomiyaensis*	*D. profundi*	F124T	F142T	F156T	F218T	F195T	*F. alba*	*K. sedentarius*	*L. mongoliensis*	*R. suwonensis*	*T. marinus*
B. hedensis	95.64																	
C. indicus	94.78	95.42																
D. terragena	96.98	94.23	94.22															
D. abyssi	94.92	94.61	96.12	94.13														
D. barathri	95.06	94.75	96.26	94.14	99.86													
D. nishinomiyaensis	94.56	94.59	95.60	93.78	98.56	98.63												
D. profundi	94.99	94.67	96.18	94.13	99.80	99.93	98.63											
F124T	94.72	94.71	95.77	93.69	98.73	98.80	99.79	98.73										
F142T	95.10	95.10	96.00	94.18	98.78	98.78	99.35	98.71	99.57									
F156T	94.86	94.86	95.91	93.91	98.94	98.94	99.50	98.87	99.72	99.86								
F218T	94.16	94.09	95.34	93.06	98.04	98.11	99.09	98.04	99.44	99.07	99.15							
F195T	94.97	95.04	95.74	94.17	98.69	98.77	99.71	98.69	99.78	99.64	99.78	99.14						
F. alba	93.66	94.60	94.78	93.10	94.44	94.44	94.56	94.36	94.57	94.53	94.79	94.02	94.32					
K. sedentarius	93.19	91.34	93.92	92.97	94.75	94.75	94.53	94.68	94.65	94.75	94.72	93.79	94.61	92.91				
L. mongoliensis	93.76	93.89	94.49	93.93	96.46	96.46	96.31	96.38	96.20	96.02	96.48	94.82	95.61	94.91	92.96			
R. suwonensis	94.26	95.05	95.24	93.53	94.83	94.90	95.33	94.90	95.61	95.55	95.47	95.10	95.64	95.98	93.32	94.90		
T. marinus	99.02	95.39	94.68	96.77	94.58	94.72	94.28	94.64	94.50	94.92	94.64	93.86	94.92	93.53	92.84	94.30	94.19	
Y. lutea	94.18	93.95	97.47	93.74	95.70	95.85	95.56	95.85	95.62	95.63	95.77	94.94	95.49	94.96	92.79	94.95	95.33	94.10

Dietzia test strains

	A. subflavus	*C. diphtheriae*	F130T	*D. aerolata*	*D. alimentaria*	*D. aurantiaca*	*D. cercidiphylli*	*D. cinnamea*	*D. kunjamensis*	*D. lutea*	*D. maris*	*D. natronolimnaea*	*D. papillomatosis*	*D. psychralcaliphila*	*D. schimae*	*D. timorensis*	*G. bronchialis*	*H. altamirensis*	*M. brevis*	*M. tuberculosis*	*N. asteroides*	*R. rhodochrous*	*S. rotundus*	*S. piniformis*	*S. niigatensis*	*T. biformata*	*T. paurometabola*	*T. otitidis*
C. diphtheriae	90.14																											
F130T	91.94	91.08																										
D. aerolata	91.97	91.85	98.41																									
D. alimentaria	91.46	90.86	98.10	98.12																								
D. aurantiaca	91.91	92.07	98.10	99.28	98.27																							
D. cercidiphylli	91.54	91.04	99.70	98.26	97.55	97.97																						
D. cinnamea	92.19	91.83	97.68	98.12	96.72	97.83	97.45																					
D. kunjamensis	91.63	91.15	97.69	97.84	97.94	97.69	97.36	97.47																				
D. lutea	92.24	91.44	98.55	98.62	98.28	98.62	98.63	98.49	97.92																			
D. maris	92.40	92.12	98.29	98.63	98.05	98.49	98.26	98.22	99.10	98.71																		
D. natronolimnaea	91.51	91.05	99.63	98.12	97.34	97.76	99.23	97.37	97.27	98.13	97.94																	
D. papillomatosis	90.87	90.58	96.26	96.67	96.04	96.53	95.88	98.33	95.78	96.91	96.51	95.81																
D. psychralcaliphila	90.93	90.03	98.81	97.47	96.72	97.19	97.90	96.01	96.58	97.12	96.93	98.01	94.91															
D. schimae	92.40	91.97	98.21	98.70	97.98	98.56	98.21	97.97	99.03	98.63	99.80	97.87	96.44	96.86														
D. timorensis	92.33	92.08	96.57	97.25	95.80	96.68	96.48	97.10	96.07	97.19	96.90	96.28	95.47	95.32	96.83													
G. bronchialis	93.35	91.67	92.16	92.98	91.76	92.99	92.45	93.27	92.18	93.07	93.06	92.21	91.75	91.26	93.13	93.51												
H. altamirensis	99.77	90.22	91.93	92.31	91.72	92.17	91.67	92.23	91.86	92.41	92.46	91.77	91.25	91.40	92.38	92.60	93.43											
M. brevis	93.61	92.12	92.83	93.61	92.51	93.55	92.95	93.63	93.14	93.35	94.07	92.89	92.09	92.13	93.82	93.78	95.88	93.81										
M. tuberculosis	94.52	91.86	91.14	91.61	90.86	91.55	91.32	92.42	91.63	91.49	92.59	91.25	90.64	90.44	92.32	92.07	93.28	94.52	94.11									
N. asteroides	93.18	90.87	91.70	92.46	91.81	92.32	92.13	93.69	92.36	92.49	93.23	91.91	92.16	90.89	93.06	92.88	93.80	93.35	95.23	93.55								
R. rhodochrous	93.66	91.75	93.89	94.29	93.42	94.01	94.01	94.74	93.93	94.45	94.73	93.87	93.16	93.14	94.68	94.82	94.56	93.82	95.49	92.78	94.36							
S. rotundus	90.77	89.86	92.01	92.25	91.47	92.26	91.96	92.31	92.24	92.06	93.06	91.72	90.84	91.05	93.06	91.71	92.01	91.02	93.26	90.75	92.69	93.52						
S. piniformis	92.99	91.46	92.23	92.75	91.82	92.55	92.38	93.56	92.09	92.80	92.96	92.20	92.25	91.31	92.91	93.24	95.58	93.13	95.71	93.00	93.95	94.78	92.37					
S. niigatensis	92.67	91.17	91.93	92.11	91.80	92.12	92.03	93.28	91.97	92.50	92.67	91.99	92.03	91.14	92.67	92.54	93.90	92.60	93.75	92.47	94.56	94.82	93.08	94.50				
T. biformata	92.77	92.39	92.74	93.18	92.52	93.05	92.78	93.26	92.99	93.06	93.64	92.54	91.95	91.72	93.57	93.43	93.88	93.12	94.82	93.18	94.62	95.05	92.48	94.01	93.67			
T. paurometabola	93.08	92.04	94.27	94.59	94.01	94.59	94.41	95.32	94.50	94.68	95.39	94.09	93.65	93.32	95.42	94.91	93.40	93.22	94.39	92.81	94.05	94.61	93.13	93.05	92.81	94.12		
T. otitidis	89.40	90.50	90.14	89.96	89.52	89.68	89.46	89.87	89.14	89.65	89.92	89.54	88.66	88.79	89.84	89.94	89.52	89.92	89.21	89.12	88.98	88.89	87.94	89.23	89.13	88.36	88.94	
W. muralis	92.82	90.71	91.10	91.81	90.84	91.75	91.21	93.09	91.36	91.84	92.23	91.09	91.60	90.17	92.16	92.33	95.29	93.04	95.00	92.52	94.79	94.98	92.03	94.11	94.00	93.08	93.61	88.86

***Leifsonia* test strains**

	A. jenensis	F96T	*L. antarctica*	*L. aquatica*	*L. bigeumensis*	*L. cynodontis*	*L. kafniensis*	*L. kribbensis*	*L. lichenia*	*L. naganoensis*	*L. pindariensis*	*L. poae*	*L. psychrotolerans*	*L. rubra*	*L. shinshuensis*	*L. soli*	*L. xyli subsp. cynodontis*	*M. lacticum*	*M. viridarii*	*O. fritillariae*	*P. flavus*
F96T	94.94																				
L. antarctica	95.92	95.75																			
L. aquatica	94.09	98.15	94.71																		
L. bigeumensis	94.19	95.34	96.06	95.23																	
L. cynodontis	94.29	98.61	95.47	98.34	96.07																
L. kafniensis	96.73	95.89	97.62	95.19	96.14	95.82															
L. kribbensis	95.70	94.55	95.06	95.56	95.2	95.48	96.39														
L. lichenia	95.16	99.32	96.23	98.00	96.73	98.41	96.57	95.59													
L. naganoensis	94.76	98.45	95.52	99.24	95.82	98.88	95.86	96.01	98.54												
L. pindariensis	95.30	95.41	97.11	94.92	95.86	95.76	97.44	95.21	96.10	95.17											
L. poae	95.45	98.38	96.40	97.66	95.95	97.78	96.81	94.54	99.11	98.06	96.67										
L. psychrotolerans	96.01	96.07	97.86	95.55	96.68	96.15	99.04	96.38	96.67	96.15	97.94	96.96									
L. rubra	94.15	94.90	96.10	94.79	95.72	94.73	96.49	94.72	95.28	95.53	95.23	95.60	96.46								
L. shinshuensis	94.69	99.32	95.45	97.93	95.96	99.09	95.65	95.15	98.96	98.47	95.10	98.41	95.85	94.64							
L. soli	94.84	99.85	96.03	98.43	97.01	98.58	96.40	95.89	99.55	98.73	96.11	99.03	96.48	95.37	99.48						
L. xyli subsp. cynodontis	94.29	98.61	95.47	98.34	96.07	100.00	95.82	95.48	98.41	98.88	95.76	97.78	96.15	94.73	99.09	98.58					
M. lacticum	95.11	94.74	94.56	94.50	93.84	94.70	95.85	95.14	94.74	95.24	94.55	95.10	96.09	93.94	94.55	94.61	94.70				
M. viridarii	95.69	95.88	97.77	95.11	95.83	95.74	97.98	95.12	96.06	95.63	99.86	96.58	98.08	95.82	95.56	96.11	95.74	95.20			
O. fritillariae	95.92	95.45	95.87	94.70	94.52	94.77	96.88	94.97	95.87	95.50	95.55	96.31	96.82	95.01	95.09	95.65	94.77	96.12	96.22		
P. flavus	94.55	95.20	95.82	94.76	93.91	94.97	96.36	94.91	95.60	95.50	95.14	95.36	96.60	95.29	94.96	95.58	94.97	95.50	95.81	97.47	
R. vestalii	93.54	94.11	95.40	94.33	95.53	94.19	95.82	94.50	94.68	94.83	95.05	94.97	95.95	99.44	93.91	95.00	94.19	93.19	95.27	94.39	94.67

***Rhodococcus* test strains** (part 1)

	C. diphtheriae	*G.bronchialis*	*M. brevis*	*N. asteroides*	F42M	F152M	*R. aetherivorans*	*R. artemisiae*	*R. baikonurensis*	*R. cercidiphylli*	*R. coprophilus*	*R. corynebacterioides*	*R. equi*	*R. erythropolis*	*R. fascians*	*R. globerulus*	*R. gordoniae*	*R. imtechensis*	*R. jialingiae*	*R. jostii*	*R. koreensis*
G.bronchialis	91.75																				
M. brevis	91.59	95.75																			
N. asteroides	90.68	93.80	95.08																		
F42M	89.66	92.23	92.9	93.47																	
F152M	89.42	91.85	93.43	93.99	95.44																
R. aetherivorans	91.30	94.52	95.38	94.52	94.16	93.41															
R. artemisiae	91.13	94.08	95.21	94.15	94.78	93.51	96.94														
R. baikonurensis	91.00	93.32	94.73	95.32	96.88	98.32	95.11	95.02													
R. cercidiphylli	91.03	93.55	94.37	94.99	94.17	94.91	95.17	94.23	96.34												
R. coprophilus	91.61	93.37	95.24	95.22	95.80	94.64	96.68	97.36	96.07	95.39											
R. corynebacterioides	91.24	94.41	95.97	95.71	94.83	94.87	96.59	95.69	96.36	96.01	96.04										
R. equi	91.24	94.44	95.11	96.17	94.44	94.64	96.09	95.25	96.21	95.86	95.86	96.93									
R. erythropolis	91.45	93.55	94.90	96.10	96.33	97.92	95.88	95.65	99.18	96.83	96.81	97.06	96.33								
R. fascians	90.94	92.62	94.36	94.68	94.51	95.20	94.52	93.80	96.59	98.72	95.22	95.89	95.29	96.49							
R. globerulus	91.46	93.61	94.69	95.22	95.51	97.44	95.09	94.82	98.89	96.56	95.79	96.52	96.53	98.44	96.12						
R. gordoniae	91.69	94.22	95.14	94.64	94.57	93.89	96.91	97.93	95.47	95.01	96.92	96.08	96.12	96.05	94.12	95.20					
R. imtechensis	91.54	93.00	93.95	94.83	93.95	95.36	94.82	94.41	96.74	95.41	95.93	95.58	97.36	97.36	95.93	97.28	95.58				
R. jialingiae	90.74	93.08	94.07	94.89	95.76	98.56	94.67	94.74	99.78	95.82	95.93	95.92	96.00	98.78	96.28	98.44	95.00	96.30			
R. jostii	90.96	93.22	94.68	94.91	94.89	95.67	95.49	94.75	97.10	96.38	96.19	96.47	96.59	97.62	96.19	98.02	95.20	97.79	96.62		
R. koreensis	92.43	94.02	95.44	95.71	95.10	95.76	95.90	95.78	97.18	96.85	96.38	96.74	97.54	97.61	96.68	97.68	95.51	98.91	96.67	98.09	
R. kroppenstedtii	87.84	91.52	92.25	91.94	91.51	92.47	94.21	92.27	94.28	93.24	92.20	96.04	93.58	93.22	93.83	92.68	93.40	92.41	92.75	92.65	92.95

***Rhodococcus* test strains** (part 2)

	C. diphtheriae	*G.bronchialis*	*M. brevis*	*N. asteroides*	F42M	F152M	*R. aetherivorans*	*R. artemisiae*	*R. baikonurensis*	*R. cercidiphylli*	*R. coprophilus*	*R. corynebacterioides*	*R. equi*	*R. erythropolis*	*R. fascians*	*R. globerulus*	*R. gordoniae*	*R. imtechensis*	*R. jialingiae*	*R. jostii*	*R. koreensis*
R. kunmingensis	91.50	93.38	94.20	96.83	94.00	94.56	95.92	94.70	96.20	96.26	95.28	96.55	98.10	96.47	95.57	96.55	95.73	96.76	96.54	96.18	96.90
R. kyotonensis	90.82	93.71	94.20	94.76	94.57	94.96	95.05	94.21	96.44	97.76	95.30	95.41	95.37	96.65	97.86	96.29	95.17	95.96	96.82	96.15	96.13
R. maanshanensis	91.10	92.98	94.81	95.46	95.17	95.55	95.53	94.89	97.01	96.46	96.74	95.95	96.10	97.80	96.28	97.02	95.42	97.31	97.31	97.87	97.52
R. marinonascens	91.72	93.82	95.16	95.82	95.37	96.16	95.94	95.50	97.55	96.28	97.08	96.51	96.94	98.10	96.13	97.68	96.26	97.83	97.28	98.23	97.95
R. nanhaiensis	91.53	93.50	94.98	95.42	94.91	95.56	96.37	95.70	97.04	95.57	96.14	96.14	96.92	97.07	95.08	96.86	96.77	97.43	97.36	96.84	97.50
R. opacus	91.88	93.85	95.32	95.98	94.58	95.36	95.89	95.46	96.81	96.16	96.41	96.80	97.69	97.42	95.99	97.48	96.07	98.92	96.42	98.37	98.77
R. percolatus	91.46	93.81	95.28	95.71	94.81	95.72	95.48	95.14	97.00	96.23	96.37	96.40	97.13	97.54	96.10	97.60	95.61	98.25	96.46	98.51	98.49
R. phenolicus	91.56	94.06	95.49	94.55	95.36	94.25	97.03	97.46	95.85	95.28	97.54	96.88	96.59	96.15	95.08	95.72	96.98	96.23	95.94	95.99	97.02
R. pyridinovorans	91.15	93.69	95.16	94.42	94.77	93.35	97.27	98.44	94.87	94.38	97.55	95.92	95.49	95.76	94.12	94.68	99.07	94.77	94.55	94.68	95.17
R. qingshengii	91.22	93.58	94.58	95.40	96.29	98.56	95.21	95.13	99.78	96.34	96.19	96.44	96.26	99.05	96.28	98.70	95.54	96.82	99.80	97.14	97.19
R. rhodnii	92.08	94.64	95.78	95.02	93.82	94.24	97.18	95.65	95.77	95.46	96.00	96.80	96.13	96.53	95.28	95.99	96.49	95.79	95.58	96.11	96.11
R. rhodochrous	91.44	94.02	95.15	94.44	95.51	94.16	97.02	97.76	95.62	94.67	97.63	96.30	96.32	96.05	94.44	95.36	98.49	95.44	95.84	95.21	95.68
R. ruber	91.13	93.78	95.11	94.46	94.65	93.44	99.49	97.16	95.03	94.56	97.23	96.59	95.80	95.73	94.31	94.97	97.13	95.33	94.92	95.23	95.63
R. triatomae	91.52	94.58	96.01	96.02	94.79	95.56	96.88	95.94	97.01	96.28	96.15	98.11	97.32	97.59	96.29	96.98	96.79	96.23	96.51	97.00	97.47
R. tukisamuensis	90.49	92.79	94.21	94.43	94.67	95.20	95.13	94.05	96.26	95.54	95.61	95.80	96.15	96.90	95.77	96.81	94.93	96.69	96.54	97.35	96.88
R. wratislaviensis	92.22	93.96	95.44	96.26	94.76	95.60	96.12	95.64	97.03	96.37	96.59	97.01	98.02	97.68	96.18	97.55	96.29	99.32	96.53	98.23	99.19
R. yunnanensis	91.50	93.92	94.73	95.35	94.53	95.35	95.10	94.52	96.74	98.90	95.42	96.45	96.37	97.13	98.37	96.99	94.99	96.66	96.38	96.92	97.07
R. zopfii	91.70	94.17	95.37	94.69	95.59	94.24	97.63	98.17	95.77	94.93	98.03	96.67	96.45	96.31	94.69	95.71	97.01	96.20	95.37	95.98	97.15
S. piniformis	91.11	95.45	95.65	94.20	92.85	92.22	94.68	94.20	93.75	94.58	94.46	95.10	95.21	94.05	93.69	94.13	94.44	93.67	93.25	93.78	94.42
S. niigatensis	91.13	93.77	93.55	94.57	93.35	92.31	94.63	94.64	93.91	93.64	94.01	94.86	95.91	94.41	93.18	94.54	94.82	94.64	94.38	94.47	95.13
W. muralis	90.45	95.29	94.86	94.58	93.56	92.05	94.15	94.38	93.76	93.66	93.84	94.66	95.55	93.90	93.25	93.84	94.34	94.18	93.42	93.88	94.79

***Rhodococcus* test strains** (part 3)

	R. kroppenstedtii	*R. kunmingensis*	*R. kyotonensis*	*R. maanshanensis*	*R. marinonascens*	*R. nanhaiensis*	*R. opacus*	*R. percolatus*	*R. phenolicus*	*R. pyridinovorans*	*R. qingshengii*	*R. rhodnii*	*R. rhodochrous*	*R. ruber*	*R. triatomae*	*R. tukisamuensis*	*R. wratislaviensis*	*R. yunnanensis*	*R. zopfii*	*S. piniformis*	*S. niigatensis*
R. kunmingensis	94.57																				
R. kyotonensis	93.61	95.95																			
R. maanshanensis	93.83	95.75	96.31																		
R. marinonascens	92.87	96.40	96.23	98.01																	
R. nanhaiensis	94.70	96.42	95.52	97.34	97.93																
R. opacus	92.89	97.25	96.01	97.66	98.57	97.93															
R. percolatus	92.48	96.65	96.13	97.78	98.42	97.40	99.25														
R. phenolicus	95.85	95.79	95.16	95.85	96.37	96.16	96.73	96.27													
R. pyridinovorans	92.07	95.13	94.54	95.24	95.41	95.71	95.23	94.92	96.96												
R. qingshengii	93.13	96.54	96.69	97.31	97.48	97.36	96.87	96.99	95.94	95.07											
R. rhodnii	92.96	96.41	95.79	96.03	96.32	95.93	96.40	96.09	96.59	96.18	95.84										
R. rhodochrous	92.71	95.77	95.37	95.32	95.98	96.07	95.91	95.60	97.46	98.98	95.84	96.52									
R. ruber	92.68	95.56	94.87	95.39	95.72	96.14	95.80	95.42	97.47	97.69	95.17	96.74	97.49								
R. triatomae	94.43	97.68	95.95	96.52	96.97	96.93	97.53	97.06	97.31	96.44	97.03	97.94	96.69	96.57							
R. tukisamuensis	93.75	95.93	95.49	97.77	97.35	96.33	97.35	97.11	95.88	94.51	96.54	95.60	94.93	94.93	96.47						
R. wratislaviensis	93.10	97.46	96.20	97.59	98.50	98.14	99.59	99.04	96.95	95.45	97.06	96.46	96.09	95.98	97.74	97.33					
R. yunnanensis	93.28	96.61	98.56	96.60	96.71	95.93	96.72	96.76	95.21	94.60	96.78	95.83	95.33	94.73	96.71	95.88	96.93				
R. zopfii	92.89	95.42	95.27	95.89	96.58	96.21	96.66	96.30	98.77	97.43	95.89	96.80	97.81	97.96	96.99	95.35	96.88	95.15			
S. piniformis	91.84	94.42	94.18	93.60	94.45	94.05	94.27	93.82	94.70	94.42	93.75	95.01	94.71	94.53	95.13	93.35	94.35	94.46	94.63		
S. niigatensis	92.10	95.49	93.41	94.04	95.02	94.28	95.10	94.65	94.92	94.65	94.31	94.61	94.96	94.42	95.38	94.28	95.20	94.09	95.00	94.43	
W. muralis	91.21	94.64	93.45	93.19	94.10	93.85	94.52	94.33	94.71	94.05	93.68	94.12	94.50	94.39	94.78	92.66	94.87	94.11	94.94	93.97	93.87